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Simultaneous Activation of PKA and Epac is Cardioprotective in Neonatal and Adult hearts

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Abstract

Cardiac vulnerability to ischaemia/reperfusion (I/R) changes during development; 14-day old rat heart being least vulnerable compared to adult or younger postnatal hearts. The underlying mechanisms are not known, thus design of age-specific optimal cardioprotective interventions is difficult. Recent work has shown activation of cAMP/PKA/Epac signalling pathway is cardioprotective in adult hearts. Whether this also mediate protection in postnatal hearts is unknown. This will be addressed in this thesis.

Wistar rats of 7, 14, and 28 days postnatal & adults were used. Cardiac proteome was examined using TMT-MS/MS and bioinformatic analysis. Langendorff perfused hearts were exposed to I/R and injury assessed by measuring cardiac enzyme release and infarction by staining with tetrazolium chloride. Isolated cardiomyocytes were used in suspension to study simulated reperfusion injury (H_2O_2 & 3mM Ca^{2+}) and viability measured by Trypan blue staining. Superfused field stimulated isolated cardiomyocytes were exposed to metabolic inhibition and contraction measured using edge tracking whilst Ca^{2+} transients measured using Fura-2-AM. Isolated mitochondria were used to study mitochondrial permeability transition pore (MPTP) sensitivity to Ca^{2+}. Selective cAMP analogues were used to directly activate PKA, Epac or both. Isoproterenol a β-Adrenergic receptor (β-AR) agonist, was used to stimulate cAMP/PKA/Epac signalling pathways.

Proteomics showed age-related variation in networks concerned with β-adrenergic signalling, Ca^{2+} homeostasis, mitochondrial regulation, and antioxidant activity. Simultaneous activation of PKA & Epac conferred cardioprotection in whole heart and in cardiomyocytes across the age ranges. In mitochondria from both adult and 14-day hearts, perfusion with isoprenaline or activation of both PKA and Epac reduced MPTP sensitivity to Ca^{2+}.

Despite significant molecular and cellular developmental changes and vulnerability to I/R, direct or indirect β-AR signalling involving Epac and PKA activation protects against acute injury in both adult and immature hearts and cardiomyocytes and this is linked to reduced MPTP sensitivity to Ca^{2+}.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of Publications & Presentations resulting from this thesis

Published Papers

- **Lewis, Martin**; Szobi, Adrian; Balaska, Dirki; Khaliulin, Igor; Adameova, Adriana; Griffiths, Elinor; Orchard, Clive H.; Suleiman, M. Saadeh. Consecutive isoproterenol and adenosine treatment confers marked protection against reperfusion injury in adult but not in immature heart: A role for glycogen. *International Journal of Molecular Sciences.* 2018; Vol. 19(2), 494; doi:10.3390/ijms19020494

Abstract Publications

- **Martin Lewis**, Adrian Szobi, Adriana Adameova, Igor Khaliulin, MS Suleiman "Consecutive treatment with isoproterenol and adenosine protects adult but not immature heart against ischaemia and reperfusion." Proc Physiol Soc 2016, 37, PCB003
- Kim Summers, **Martin Lewis**, Hua Lin, Sarah George, MS Suleiman. Changes in cardiac survival signaling during postnatal development: implications for cardioprotection. J Mol Cell Cardiol 2016, 97: 1-18
- Kim Summers, **Martin Lewis**, Hua Lin, Sarah George, MS Suleiman. Changes in survival signalling-related cardiac phosphoproteome during postnatal development: Implications for vulnerability to Ischemia/Reperfusion. Curr Res Cardiol 2016, 3(3): 89-116
- **Lewis M**, Hall J, Keylock L, et al. P15 Cyclosporine A is protective against oxidative stress in adult but not in immature isolated cardiomyocytes. Heart 2018;104: A7

Oral Presentations

- **Martin Lewis**, Kate Heesom, Clive Orchard, M.S. Suleiman. Age- Related Variation in the Cardiac Proteome of Proteins Involved in Signalling and Response to Ischaemia. International Academy of Cardiovascular Sciences, North American Meeting, Sherbrooke, Quebec, Canada September 2016
- **Martin Lewis** The susceptibility of the developing myocardium to cardiac insults Association of Paediatric Anaesthetists of Great Britain & Ireland Annual Scientific Meeting June 2017, Bristol, UK
- **M. Lewis**, K. Heesom, C. Orchard, M.S. Suleiman Age- Related Proteomic Changes in the Developing Rat Heart. 5th International Congress on Analytical Proteomics, July 2017, Caparica, Portugal
- **Martin Lewis**, Katie Hall, Igor Khaliulin & M.S. Suleiman Developmental changes in Mitochondrial Permeability Transition: Implications for Cardioprotection by cAMP signalling IACS-ES 2018, Smolenice, Hungary

Poster Presentations (not otherwise listed above)

- **Martin Lewis**, Kieran Smith & M.-Saadeh Suleiman Selective activation of PKA & EPAC produces resistance to simulated ischaemic injury in freshly isolated cardiomyocytes. IACS-ES 2017, Pecs, Hungary
- **M.J. Lewis**, K. Hall, I. Khaliulin & M.S. Suleiman PKA and Epac Agonists Ameliorate Mitochondrial Sensitivity to Ischaemia/Reperfusion Injury in Adults & Immature Hearts. Association for Cardiothoracic Anaesthesia and Critical Care Annual Scientific Meeting 2018, Bristol, UK

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- September 2016- $500 awarded for nomination for James Willerson Young Investigator Prize at International Academy for Cardiovascular Sciences meeting, Sherbrooke, Quebec, Canada
- March 2017- €500 travel award to V-ICAP 2017 Proteomics Conference, Lisbon, Portugal
- May 2018- €250 Early Career Investigator award at IACS-ES 2018, Smolenice, Slovakia for oral presentation
- June 2018- £50 poster prize for presentation at ACTACC 2018 meeting in Bristol, UK

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List of Abbreviations

EPAC	**Exchange Protein Activated by cAMP**
6-Bnz	**6-Bnz-cAMP-AM**
8-Br	**8-Br-cAMP-AM**
AC	**Adenylate Cyclase**
ACC	**acetyl CoA Carboxylase**
Aden	**Adenosine**
ADP	**Adenosine Diphosphate**
AKAP	**A- kinase anchoring protein**
ANT	**Adenine Nucleotide Translocase**
ATP	**Adenosine Triphosphate**
CaMKII	**Calcium/calmodulin- dependent Protein Kinase II**
cAMP	**Cyclic Adenosine Monophosphate**
CFR	**Coronary Flow Rate**
CICR	**Calcium Induced Calcium Release**
CK	**Creatine Kinase**
CPP	**Coronary Perfusion Pressure**
CPT	**Carnitine palmitoyltransferase**
CPT	**8-CPT-2'-O-Me-cAMP-AM**
CsA	**Cyclosporin A**
CVR	**Coronary Vascular Resistance**
CypD	**Cyclophilin D**
ECC	**Excitation- Contraction Coupling**
FAD	**Flavin Adenine Dinucleotide**
GSK	**Gycogen Synthase Kinase**
G6P	**Glucose- 6- Phosphate**
GO	**Gene Ontology**
GPCR	**G- protein coupled receptor**
GTP	**Guanosine Triphosphate**
HKII	**Hexokinase II**
I/R	**Ischaemia/ Reperfusion**
IMM	**Inner Mitochondrial Membrane**
IMS	**Mitochondrial Intermembrane Space**
Iso	**Isoprenaline/ Isoproterenol**
LC	**Liquid Chromatography**
LDH	**Lactate Dehydrogenase**
LTCC	**L- type Calcium Channel**
LV	**Left Ventricle**
LVDP	**Left Ventricular Developed Pressure**
LVEDP	**Left Ventricular End Diastolic Pressure**
MCD	**Malonyl- CoA Decarboxylase**
MPC	**Mitochondrial Pyruvate Carrier**

MPTP	**Mitochondrial Permeability Transition Pore**
MS	**Mass Spectrometry**
NAD	**Nicotinamide Adenine Dinucleotide**
NCX	**Sodium- Calcium Exchanger**
NHE	**Sodium- Hydrogen Exchanger**
OMM	**Outer Mitochondrial Membrane**
PDH	**Pyruvate Dehydrogenase**
PDK	**Pyruvate Dehydrogenase Kinase**
PiC	**Inorganic Phosphate Carrier**
PKA	**Protein Kinase A**
PKB	**Protein Kinase B**
PKC	**Protein Kinase C**
PLB	**Phospholamban**
RIPA	**Radioimmunoprecipitation assay (buffer)**
ROS	**Reactive Oxygen Species**
RyR	**Ryandine Receptor**
SAN	**Sinoatrial node**
SERCA	**Sarcoplasmic Reticulum Ca2+-ATPase**
SOD	**Superoxide Dismutase**
SR	**Sarcoplasmic Reticulum**
TCA	**Tricarboxylic Acid Cycle**
TMT	**Tandem Mass Tag**
TnI	**Troponin I**
TP	**Temperature Preconditioning**
TTC	**Triphenyl Tetrazolium Chloride**
VDAC	**Voltage Dependent Anion Channel**
β-AR	**β-Adrenoreceptor**

1 General Introduction

This thesis examines “The Cardioprotective Role of the Exchange Protein Activated by cAMP (Epac) during Postnatal Development”. The introduction addresses this by first examining cardiac metabolism and physiology in the healthy adult heart, and then looking at how those processes become disordered in ischaemia and in subsequent reperfusion, with particular emphasis on the key role of the mitochondria in that process. It then moves on to look at different modes in which the heart has historically been protected from that injury before looking at how cAMP signalling through the β- adrenoreceptor, particularly through EPAC and its counterpart PKA interacts with those targets for protection. Finally, it provides an overview of some key differences between the adult and developing heart with relevance to cardioprotection.

1.1 Overall View of Cardiac Physiology & Metabolism

The heart produces and consumes the largest amount of energy of any organ, and if that production of energy were to fail, the organism as a whole dies. The heart requires a large flux of ATP in order to support its repeated cycles of excitation and contraction; these are energy-intense functions requiring movement of ions against electrochemical gradients and conformational changes of proteins to thermodynamically unfavourable states. To that end, a large proportion of the biomass in the heart is dedicated to energy production with multiply redundant pathways (

Figure *1-1*). The heart is able to utilise a range of metabolic substrates to feed this demand for energy (Bing, Siegel et al. 1953); under physiological conditions the majority (60-90% comes from utilisation by oxidation of fatty acids (Vusse, Glatz et al. 1992), with glucose, broken down through glycolysis, the remainder (10-40%) (Gertz, Wisneski et al. 1988).

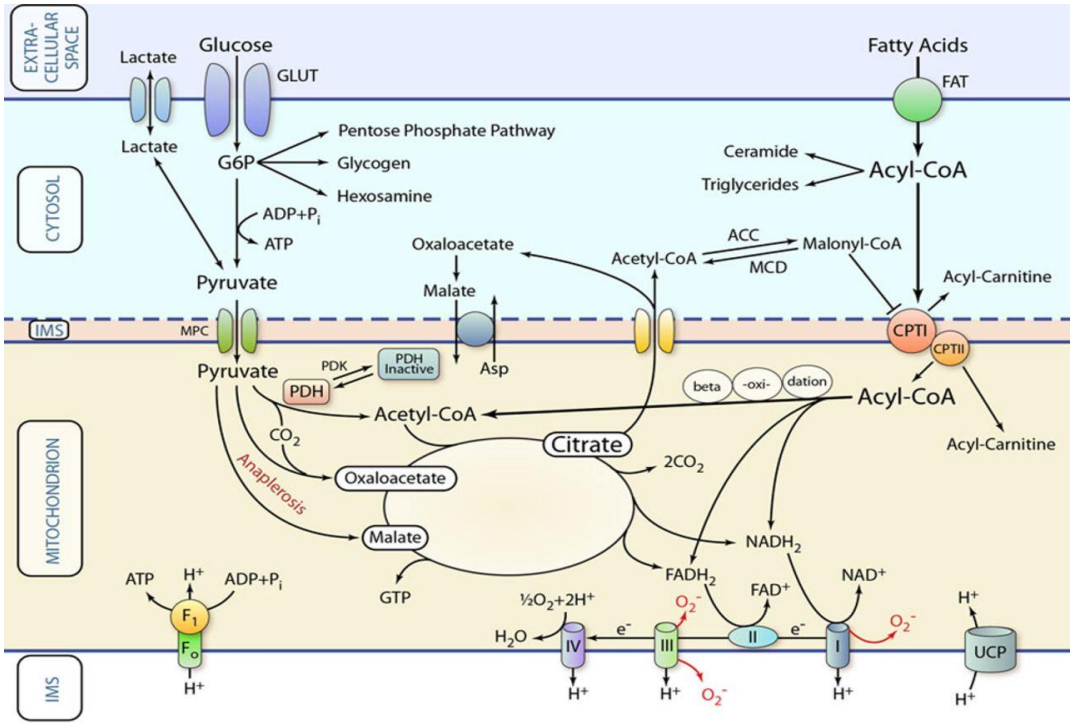


Figure 1-1 Cardiac metabolic pathways.The reducing equivalents are used by the electron transport chain to generate a proton gradient, which in turn is used for the production of ATP. ACC indicates acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; FAT, fatty acid transporter; G6P, glucose 6-phosphate; GLUT, glucose transporter; IMS, mitochondrial intermembrane space; MCD, malonyl-CoA decarboxylase; MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; and PDK, pyruvate dehydrogenase kinase (Doenst, Nguyen et al. 2013).

The tricarboxylic acid cycle (TCA) is the convergent point for metabolism of the majority of these substrates under physiological conditions (Doenst, Nguyen et al. 2013). It refers to a series of enzymatic conversions of substrates to acetyl- CoA followed by stepwise decarboxylation producing NADH and $FADH_2$ to supply mitochondrial oxidative phosphorylation, as well as GTP. NADH is an important electron donor in redox reactions in particular the generation of the mitochondrial proton gradient; the ready supply of it in the oxidised form is thus vital for physiological ATP production.

The main product of the TCA cycle is NADH, which is then used in the mitochondrial electron transport chain to produce a proton gradient. This functions through the donation of electrons through a series of inner mitochondrial membrane bound multiprotein complexes ultimately to be accepted by oxygen. It is this proton- motive force which is ultimately utilised to synthesise ATP.

Roughly 90% of the ATP production in the heart comes from substrates funnelled through the tricarboxylic acid cycle. The main substrate, fatty acids, cannot be synthesised *de novo* in the heart, although there is a limited store of triglycerides. Consequently, the heart is exquisitely sensitive to disruptions to its supply of fatty acids through blood flow as it becomes dependent upon minority substrates for maintenance of ATP production.

1.1.1 Excitation-Contraction (E-C) coupling & Ca^{2+} cycling

Excitation- contraction coupling in the heart refers to the linkage between electrical excitation of tissues and their organised, rhythmic, contraction. The heart as a whole exhibits automaticity; this generates a wave of depolarisation starting in the sinoatrial node and spreading in a synchronised manner through the conducting system of the heart before exciting the cardiomyocytes directly which causes the release of Ca^{2+}. This Ca^{2+} transient is key to the interaction between actin and myosin which produces contraction; it must be recycled back into storage for the heart to relax and so the movement of Ca^{2+} into and out of the sarcoplasm is driving the

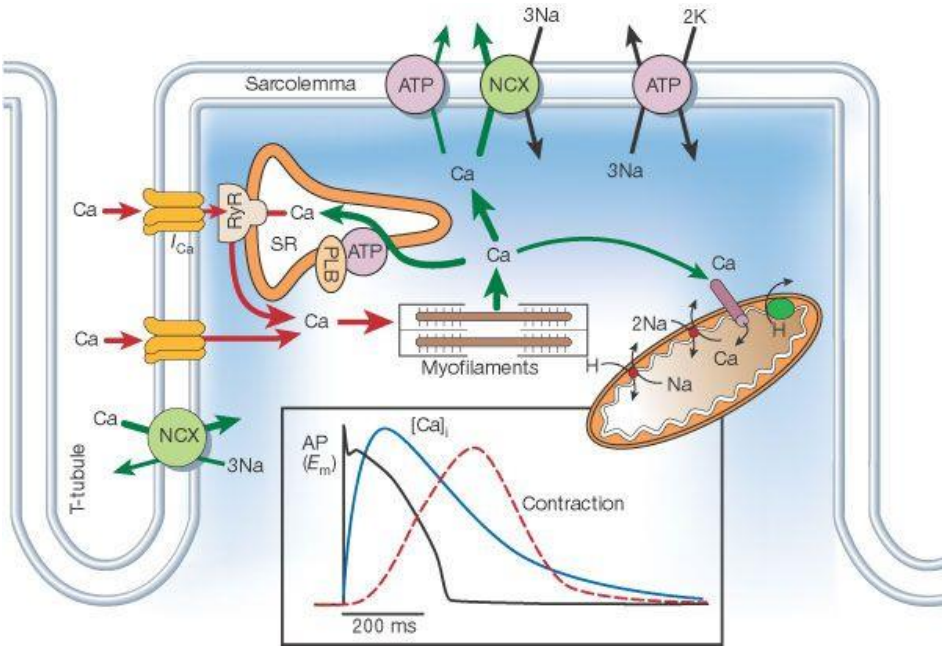


Figure 1-2 Cardiac Excitation-contraction coupling. A diagram showing processes in adult cardiomyocyte that are responsible of Ca2+ cycling and the associated contraction-relaxation cycles. The inset is a summary of timeline associated with the action potential, Ca2+ transient and contraction. NCX, Na+/Ca2+ exchange; RyR, Ryanodine receptor; SR, sarcoplasmic reticulum; ATP, ATPase; PLB, phospholamban. From Bers 2002.

mechanics of contraction (Bers 2002). A schematic overview of Ca^{2+} mobilisation during E-C coupling is shown in Figure 1-2.

1.1.1.1 Excitation

The influx of Ca^{2+} begins during the plateau phase of the cardiac action potential. In this phase, L-type Ca^{2+} channels open once the membrane potential is greater than -40 mV (Beeler and Reuter 1970, Benitah, Alvarez et al. 2010) causing an influx and rapid rise in intracellular Ca^{2+}. This inward Ca^{2+} current is relatively small; however this triggers in the adult heart a much larger release of Ca^{2+} from intracellular stores (Ca^{2+}-transient). This process is known as calcium- induced calcium release (CICR) (Fabiato 1985, Talo, Stern et al. 1990, Bers 2002), which is regulated through the action of the ryanodine receptor (RyR) family (Pessah, Waterhouse et al. 1985).

1.1.1.2 Contraction

Cardiac muscle is composed of thick and thin filaments. The thick filament is comprised of many molecules of myosin, with heads which may form crossbridges. The thin filaments however are made up of actin in helical form, with troponins and tropomyosin alongside these helices covering the myosin binding sites of actin when the muscle is relaxed. There are multiple forms of troponin; I, which inhibits the actin-myosin interaction; C, responsible for Ca^{2+} binding; and T which interacts with tropomyosin. Once troponin C binds to Ca^{2+}, it initiates a cascade of conformational changes though troponin T and I through to tropomyosin, unveiling the previously occluded myosin binding site (Yasui, Fuchs et al. 1968, Eisenberg and Kielley 1974) allowing the myosin head and actin to bind, in an ATP- consuming interaction, causing a conformational change of the head and resulting in movement of the filaments with reference to one another and thus contraction (Coupland, Pinniger et al. 2005). Once the ADP disassociates, the contractile phase is completed.

1.1.1.3 Relaxation

Synchronous with the rise in sarcoplasmic Ca^{2+}, SERCA, NCX and the mitochondrial uniporter as important determinants of Ca^{2+} homeostasis increase their action in response to the change (Hove-Madsen and Bers 1993).

The continued release of Ca^{2+} from intracellular stores triggered by CICR and regulated by the ryanodine receptor must cease in order for relaxation to occur. The termination of this is controlled through interaction of the ryanodine receptor with calsequestrin, a calcium binding protein in the sarcoplasmic reticulum. Prior to excitation, when the SR Ca^{2+} content is high, calsequestrin is unbound to the RyR, and in this state, the RyR may be induced to cause CICR by a rise in sarcoplasmic Ca^{2+}. This release of calcium reduces the concentration remaining in the SR, which then allows the calsequestrin to bind to the RyR inactivating the binding site for Ca^{2+} (Stevens, Terentyev et al. 2009), so stopping the Ca^{2+} release.

A further complex network of systems exists to regulate the levels of Ca^{2+} that are permitted to occur during each phase of the cardiac cycle, balancing inflows from SR stores and the extracellular environment. When Ca^{2+} release is triggered from the SR, the amount which is released depends upon how much is stored in the SR; i.e. the higher then content, the greater the proportionate release (Bassani, Yuan et al. 1995). Meanwhile, the LTCC in the t-tubules become inactivated during prolonged depolarisation in a Ca^{2+} dependent manner (Yue, Backx et al. 1990). By a combination of these effects, a negative feedback loop exists (Sham, Cleemann et al. 1995); the more Ca^{2+} released from the SR, the more rapid the inactivation of the LTCC, and so reducing the influx of Ca^{2+} into the cell, feeding back into the mechanism of CICR and so reducing the amount of Ca^{2+} released from the SR. Thus there is a homeostatic mechanism balancing release in a given state of the cell. However, it is not always physiologically appropriate for the Ca^{2+} level to be maintained at this set point. In situations where the cardiac output must be increased, there needs to be a positive inotropic response which is accomplished through activity at the β- adrenoreceptor. The end result of activity here is to increase Ca^{2+} transient amplitude and hence contractility, as well as increased Ca^{2+} cycling for the chronotropic response through the actions of cAMP as a second messenger from this receptor family. As is discussed later in Section 1.5, this signalling system is a vital link between the external physiological environ and control of the contractile apparatus of the cardiomyocyte.

Shortly afterwards, in phase 3 delayed rectifier K^+ channels open causing rapid repolarization; the L-type Ca^{2+} channels close as a consequence as the membrane

potential once again falls. In addition to stoppage of Ca^{2+} entry, the elevated cytosolic Ca^{2+} is removed mainly via SERCA & NCX resulting in a fall back to diastolic levels leading to dissociation of Ca^{2+} from troponin C.

1.2 Cardiac Ischaemia & Reperfusion (I/R) Injury

Ischaemia is the situation which occurs when the blood flow to a tissue is mismatched to the demand of that tissue and so is insufficient to meet the metabolic requirements. There are multiple components to this phenomenon; stagnant hypoxia, with a reduced oxygen delivery to the tissue is important, but the other components of reduced substrate delivery and impaired efflux are also significant. Reperfusion injury occurs when that blood flow is resumed; and is in itself independently associated with contractile dysfunction, intracellular hypercalcaemia, and necrotic cell death. Therefore ischaemia and reperfusion injury, as distinct phenomena, have linked but distinct mechanisms of harm.

1.2.1 Pathophysiological Basis of I/R Injury

Ischaemic events due to interruption or cessation of blood flow are a significant trigger for morbidity clinically, and affect every organ system, albeit some more commonly than others. Treatment for these events invariably involves restoration of normal flow- revascularisation, or reperfusion. This reperfusion, the reintroduction of oxygen and metabolic substrates alongside the removal of the products of metabolism is intended at normalising the situation within a tissue but triggers a series of events within the newly reperfused cells, some of which are significantly deleterious. Indeed, some therapeutic modalities in themselves invariably result in both ischaemia and reperfusion- organ allograft transplantation is a key example.

However, the more common scenario is cardiac ischaemia and reperfusion injury; within the heart this situation occurs for both pathophysiological and iatrogenic reasons. The most obvious and most frequent event in the adult is the occlusion of a coronary artery by a thrombus leading to reduced or absent flow distal to the occlusion, and subsequent insufficiency of flow to the tissues in the territory of that coronary artery.

In the setting of open-heart surgery, cardiopulmonary bypass is used in order to facilitate operating upon the interior of an arrested heart. This necessarily involves clamping of the aorta at the root and so there is no physiological flow of blood

through any of the coronary arteries, resulting in global ischaemia. This may lead to an excess risk of postoperative complication, a prolonged stay in hospital, as well as a poorer cardiac and functional outcome from surgery if the risk of I/R injury is not mitigated (Buckberg 1995).

Other situations can occur more rarely resulting in cardiac I/R injury from extra-cardiac pathologies, such as an obstructing pulmonary embolus in the right sided circulation, or from hypoxaemic hypoxia from reduced oxygen uptake for any other reason, but these scenarios are very heterogenous and so outside the scope of this work.

1.2.2 Cellular Consequences of Cardiac Ischaemia

1.2.2.1 Inhibition of Oxidative Phosphorylation

The primary mode by which ATP is produced to satisfy the energy needs of the cardiomyocyte is through oxidative phosphorylation in the mitochondria by the tricarboxylic acid cycle; this ultimately requires oxygen as an electron acceptor, so a lack of oxygen in ischaemia stops ATP production via this means. This leaves breakdown of glycogen via glycolysis as the sole means for ATP production during ischaemia; stoichiometrically this is inefficient and so ATP levels within the cardiomyocyte drop rapidly, to about 80% of the baseline 2 minutes after the onset of ischaemia (Murphy and Steenbergen 2008).

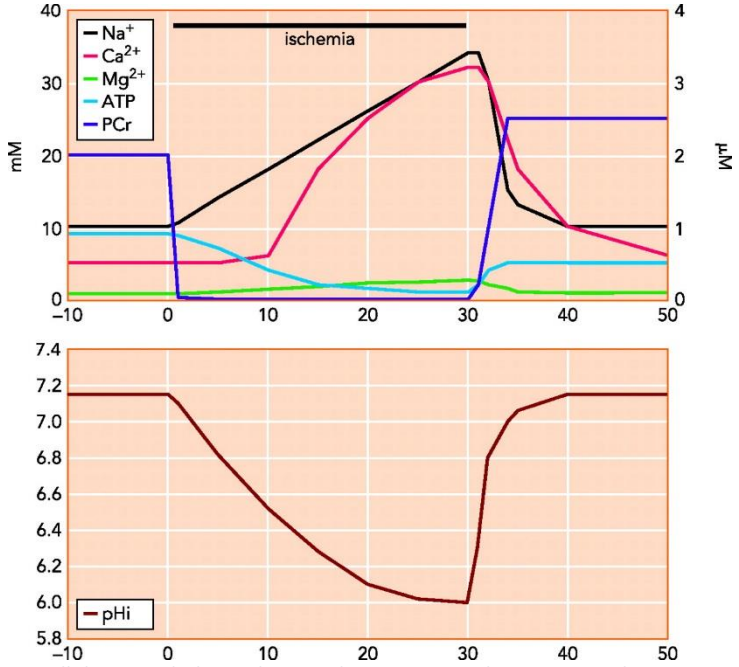


Figure 1-3 Key cellular metabolic and ionic changes in rat heart exposed to 30 min ischaemia followed by reperfusion (Murphy and Steenbergen 2008).

In this situation where oxygen and fatty acids are not readily available, glucose is broken down to form ATP and pyruvate (Taegtmeyer 1994, Taegtmeyer, Lam et al. 2016);

$$\text{Glucose} + 2\ NAD^+ + 2\ ADP + 2\ P_i \rightarrow 2\ \text{pyruvate} + 2\ NADH + 2\ H^+ + 2\ ATP + 2\ H_2O$$

Under aerobic conditions, the NADH would normally be recycled to NAD^+ through its action at complex 1. This is not possible if the electron transport chain is inhibited, and so in order to carry on with the process of ATP generation via glycolysis, NADH is instead recycled by formation of lactate;

$$\text{pyruvate} + NADH + H^+ \rightarrow \text{lactate} + NAD^+$$

- therefore further NAD^+ is available for glycolysis to continue. However, this consumes the supply of pyruvate which is then not available for entry into the TCA cycle.

So rather than pyruvate, as the product of glycolysis, being converted to Acetyl- CoA for entry into the TCA cycle it is instead used to form lactate, by lactate dehydrogenase in a step converting NADH to NAD^+. Lactate as the product of this final step readily becomes ionised and so the net effect of all energy production occurring via glycolysis is an accumulation of hydrogen ions and lactate. This results in an intracellular acidosis. The metabolic and ionic changes seen are summarised in Figure 1-3.

1.2.2.2 Results of intracellular acidosis & ionic disruption

An important mechanism for the maintenance of intracellular pH homeostasis is the sarcolemmal Na^+/H^+ exchanger. In response to the rise in H^+ ions, this antiporter increases its flux; this leads to an increase in intracellular Na^+ levels $[Na^+]_i$ (Lazdunski, Frelin et al. 1985, Silverman and Stern 1994). What might then be expected would be an increase in Na^+ extrusion through the Na^+-K^+ ATPase; however in the ischaemic low-ATP state, this pump is inhibited. Rather the transmembrane Na^+ gradient is reduced, and this has the effect of reversing the direction of the Na^+/Ca^{2+} exchanger (NCX); in the ischaemic environment the acidosis limits NCX activity, limiting the rate of rise of intracellular $[Ca^{2+}]$. Nonetheless, another reservoir into which Ca^{2+} is usually stored is the sarcoplasmic reticulum via SERCA; this is an ATP dependent process and so inhibition in the ischaemic state contributes to a rise in intracellular Ca^{2+} (Ferrari, Pedersini et al. 1993).

Ca^{2+} loading causes disruptive changes to the heart during ischaemia. However, this depends on the extent of Ca^{2+}-loading which is determined by the duration of ischaemia. Long periods of sustained ischaemia can result in sufficient Ca^{2+} loading to trigger mitochondrial permeability transition (usually more a feature during reperfusion, Section 1.2.3). Further, it can cause hypercontracture and rigor of the heart (Malinow, Batlle et al. 1953, Anmann, Eimre et al. 2005, Abdallah, Wolf et al. 2010).

1.2.3 Reperfusion of the Ischaemic Heart

Acidosis is beneficial during relatively short periods of ischaemia as it inhibits MPTP opening but it eventually becomes damaging as the ischaemic time becomes extended. Sustained low pH would trigger significant Na^+ accumulation and eventually resulting in Ca^{2+} accumulation. However, this can also occur during reperfusion following relatively short periods of ischaemia (see Figure 1-3, above). Whilst intuitively restoring normal perfusion of tissue would seem a necessary step in limiting injury, reperfusion does paradoxically cause further injury in itself, made evident by an increase in infarct size, reduction in contractility and arrhythmia in the immediate aftermath of reperfusion. Several phenomena occur which each contribute to the additional injury incurred during reperfusion, governing ionic homeostasis, pH levels, and oxidative stress.

A key early event when flow is restored is normalization of pH (Piper, Garcña-Dorado et al. 1998). The reintroduction of blood flow removes H^+ ions from the extracellular spaces, and so creates a pH gradient between the intracellular space and the intersititium. In the reperfused state, the Na^+/H^+ antiporter becomes still more active, so intracellular Na^+ levels rise whilst the pH normalizes, triggering a further rise in intracellular Ca^{2+} (Abdallah, Wolf et al. 2010) with rising ATP levels, through NCX. This excess in Ca^{2+} levels is handled by two main reserves; SR storage via SERCA, and mitochondrial Ca^{2+} uptake. This can become overwhelming and lead to mitochondrial swelling, and ultimately rupture (Allen and Orchard 1987, Griffiths, Ocampo et al. 2000)

A further consequence of the removal of the acidosis is the loss of inhibition of the contractile machinery; this has the effect of increasing ATP consumption and causing hypercontracture, and so is in itself harmful as it uses energy reserves and postpones re-establishment of ionic and metabolic homeostasis (Ladilov, Siegmund et al. 1995).

ATP production becomes re-established along with the reintroduction of oxygen and metabolic substrates, when TCA flux is again viable. It has been known for some time that some component of reperfusion injury results from the resumption of ATP

production, known as the 'oxygen paradox' (Hearse, Humphrey et al. 1973, Ganote 1983). An important element of this is oxidative stress and the production of reactive oxygen species (ROS). This occurs from reintroduction of oxygen to the mitochondrial electron transport chain complexes, and activation of xanthine and NADPH oxidases.

1.2.3.1 Reactive Oxygen Species

ROS refers to a collection of oxidants containing oxygen; these may be radical or non-radical species capable of freely oxidizing other molecules. Biologically, some significant examples of these include the superoxide radical ($O_2^{-}\cdot$), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2) forming peroxynitrite, ONOO− (Zorov, Juhaszova et al. 2014) (Figure 1-4). Consequences of the release of these species include damage to cell and organelle membranes, interruption of signalling cascades but most significantly they increase the opening likelihood of the mitochondrial permeability transition pore (detailed in section 1.3) which is the key event linking reperfusion to tissue damage.

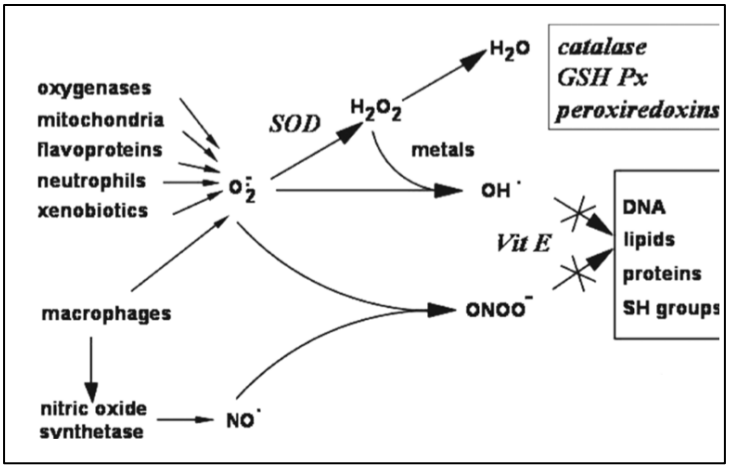


Figure 1-4 Formation and removal of reactive oxygen species (Turrens 2007)O2-·, superoxide radical; ·OH, superoxide radical; ONOO–, peroxynitrite.

Under normal circumstances, ROS are produced at a relatively low rate through mitochondrial respiration and other processes, but are dealt with through a variety of enzymatic antioxidant systems to prevent any significant damage. However, whether in a physiological state of low production or a pathological state where production is higher, the likely source in eukaryotes is the electron transport chain.

Mechanistically, this is likely because under conditions of high membrane potential and low oxygen transport, it becomes more energetically favourable for electrons to 'leak' from the chain of complexes (Ohkohchi, Endoh et al. 1999).

Thus, the term 'oxidative stress' is used to describe that situation, where the production of ROS cannot be sufficiently controlled. Many external influences on the heart can trigger this, but it is particularly marked in the forms of surgery involving ischaemia and reperfusion; that is especially in cardiac surgery requiring aortic clamping (Dhalla, Elmoselhi et al. 2000).

Given that ROS are produced endogenously however, it is unsurprising that there are enzymatic systems in place to remove them. These are concentrated spatially in the areas in which ROS are most prevalent- at the mitochondria, in the case of glutathione peroxidase and superoxide dismutase. The spatial relationship minimises the time between ROS generation and detoxification.

Superoxide dismutase, SOD, is a particularly important antioxidant (Turrens 2003). It catalyses the conversion of O2.- to hydrogen peroxide through-

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

This oxygen radical converted to hydrogen peroxide by SOD is highly reactive. Were it not dismutased to H_2O_2, an alternative reaction with NO forms the extremely potent peroxynitrite radical. Such is the importance of this enzyme that in mitochondrial SOD KO mice, huge levels of oxidative stress are seen causing death within days post-natal (Li, Huang et al. 1995). However, H_2O_2 is in itself a potentially damaging species. It is specifically removed by catalase;

$$H_2O_2 \rightarrow 2H_2O + O_2$$

-so by two successive enzymatically catalysed reactions, a harmful species is rendered much less so. The removal of H_2O_2 is also not solely dependent upon the

action of catalase; glutathione peroxidase, present in both the cytoplasm and mitochondria, may also scavenge H_2O_2, as can peroxiredoxin. Glutathione peroxidase is particularly important in preventing lipid peroxidation in organelle membranes and in removing membrane bound lipid hydroperoxides (Meister and Anderson 1983, Szymonik-Lesiuk, Czechowska et al. 2003). A number of studies have shown a protective effect of supranormal levels of glutathione peroxidase or increased flux through the reaction it catalyses; for instance increased glutathione levels, the substrate for glutathione peroxidase, are correlated with greater levels of function and reduced infarct size in a porcine model of cardiac surgery (Singh, Lee et al. 1989); this helps to demonstrate the importance of these enzymatic defence mechanisms in cardiomyocytes.

Non enzymatic mechanisms also exist which reduce the activity of ROS in the heart. These are particularly important for the detoxification of some of the very potent oxidising species such as the $ONOO^{\cdot-}$ radical. Rather than being eliminated by enzymatic activity, simpler compounds exist to quench their action; these include ubiquinone, and Vitamin E (Turrens 2003).

1.2.3.2 ROS and I/R injury

A balance exists between variable production of ROS, increased in conditions of oxidative stress, and elimination, through enzymatic and non-enzymatic mechanisms. Whilst production of ROS can occur during ischaemia through such pathways as enhanced arachidonic acid metabolism the main involvement of ROS in I/R injury comes through excess Ca^{2+} and oxidative stress on reperfusion leading to significantly increased electron leak in the mitochondria (Dhalla, Elmoselhi et al. 2000, Robin, Guzy et al. 2007). Once reperfusion has occurred, the mitochondria are in themselves impaired due to the events during ischaemia and so their ability to generate flux through the electron transport chain is reduced (Makazan, Saini et al. 2007); this leads to an accumulation of less stable intermediates and then a generation of ROS.

These ROS are a potent trigger for opening of the MPTP on restoration of blood flow. This occurs in the context of a Ca^{2+} loaded cardiomyocyte; these conditions prime the MPTP to open at the onset of reperfusion. The role of ROS in regulating the opening of the MPTP is critical to determining the ultimate degree of injury as a consequence of reperfusion. The events leading to opening of the MPTP as a consequence of ischaemia & reperfusion are summarised in Figure 1-5.

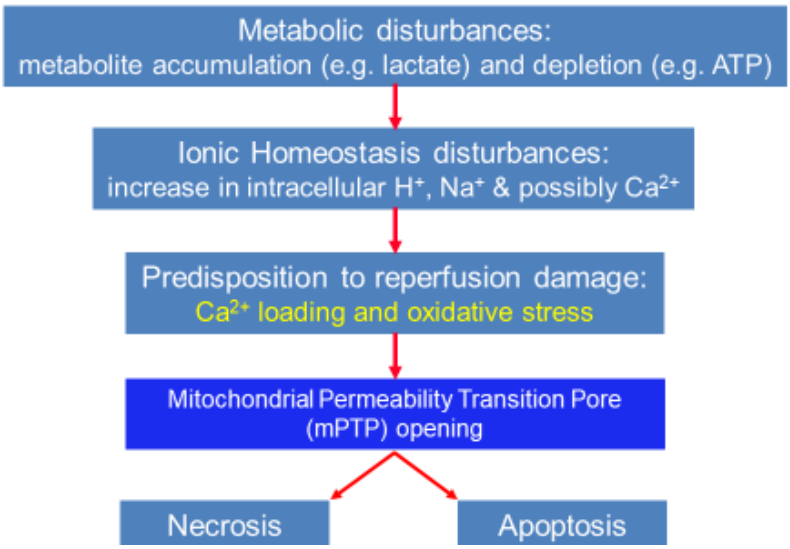


Figure 1-5 Schematic diagram showing the sequence of events thought to lead to cell death in the cardiomyocyte following ischaemia and subsequent restoration of perfusion.

Initially, cessation of blood flow leads to accumulation of metabolic byproducts from the switch to anaerobic respiration, and a depletion of ATP. This leads to a disturbance in ionic homeostasis due to failure of energy-dependent mechanisms to maintain the physiological gradients, followed as a consequence by Ca^{2+} loading and an increase in ROS production leading to MPTP opening. This is the final step towards death by apoptosis or necrosis.

1.3 The Mitochondrial Permeability Transition Pore (MPTP)

Mitochondria provide the main source of ATP in mammalian cells and are particularly dense in tissues with high energy demands and so are a prominent ultrastructural feature in the cardiomyocyte. They have two surrounding membranes; an outer and an inner mitochondrial membrane The outer mitochondrial membrane (OMM) is relatively permeable to small solutes (<5 kDa) but the inner mitochondrial membrane (IMM) is highly impermeable and provides the functional barrier to solute movement (Seifert, Ligeti et al. 2015).

In order for their energy- producing function to operate, the inner mitochondrial membrane must remain relatively impermeable. However, in certain conditions- high calcium, phosphate and especially under oxidative stress- this membrane becomes more permeable. This leads inevitably to swelling of the mitochondrion, uncoupling of respiration, reversal of the F_1/F_0 ATPase and eventually rupture (Halestrap and Richardson 2015). This phenomenon was originally known as the permeability transition; Haworth & Hunter in 1979 (Hunter and Haworth 1979) demonstrated that this was due to the formation of a non-selective channel in the inner mitochondrial membrane porous to substances < 1.5 kDa in mass. This permeability transition was inhibited by the addition of cyclosporin A, (Crompton, Ellinger et al. 1988), which provided the first direct evidence of cyclophilin-D as a structural component of the hypothetical pore.

Under conditions where the pore opens, there is no barrier to equilibration between the mitochondrial matrix and the cytosol. Thus, a consequence of pore opening is the movement of small molecules which have a collective osmotic pressure; this movement leads to the swelling of the organelle and eventual rupture. What follows is the release of membrane proteins including cytochrome c, which may precipitate an apoptotic response via Apaf-1 and the activation of procaspase-9 (Desagher and Martinou 2000).

Many factors have been noted as having influence over the opening propensity of the MPTP; predominantly these factors operate by enhancing or reducing the

sensitivity of the pore to calcium (Halestrap, McStay et al. 2002, Halestrap and Brenner 2003, Halestrap 2009); for instance cyclosporin A operates through a decrease in the pore sensitivity to calcium induced opening. Therefore, as the sensitivity can vary independently of calcium concentration, pore opening can occur without changes in ambient calcium levels.

Further observations (Szabo and Zoratti 1991, Zoratti and Szabo 1994) have previously suggested the possibility that the pore may rapidly transition between open and closed states with a range of meta-stable states in between. These studies are based upon electrophysiological observation; it is unknown whether these states exist in the physiological setting. Indeed, after induction of MPTP opening, mitochondria once imaged are seen to be either indistinguishable from the untreated appearance, or very swollen, without an intermediate phenotype.

1.3.1 Structural Components of the MPTP

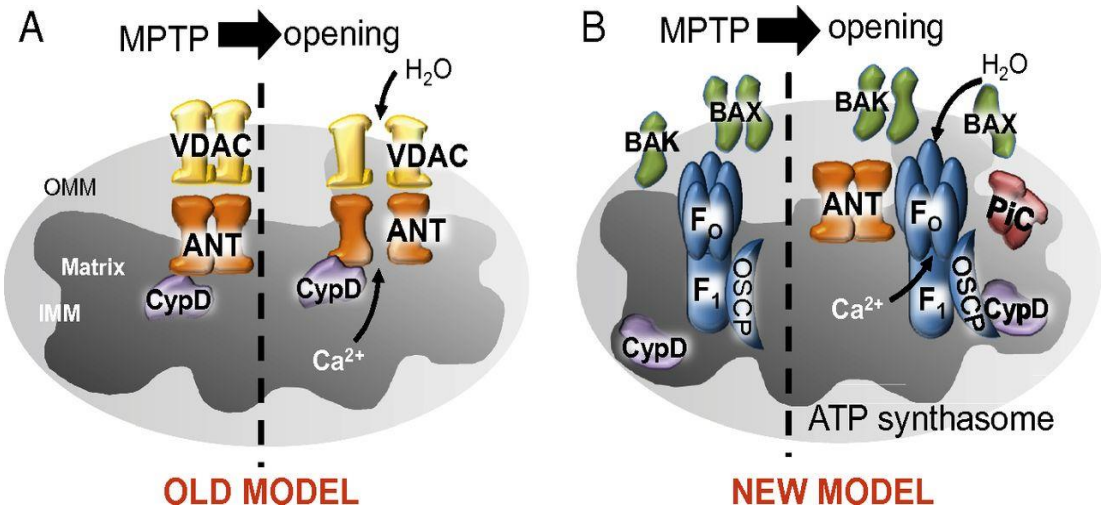


Figure 1-6 Schematic of the original model of the MPTP

(A) The MPTP was proposed to be one contiguous pore composed of VDAC and ANT regulated by CypD, which formed at contact sites between the inner and outer membranes.

(B) (B) Schematic of the new model of the MPTP consisting of Bax/Bak on the outer mitochondrial membrane to impart a level of necessary permeability and the F_1F_0 ATP synthase on the inner mitochondrial membrane regulated by CypD in the matrix as the component that responds to stimuli to initiate opening, efflux of Ca^{2+} and loss of membrane potential, and mitochondrial swelling (Karch et al. 2014)

The precise identity of the components of the MPTP remains unclear; as does much knowledge about the dynamics of the formation of the pore and indeed several groups have differing perspectives on the composition of the pore. However, several

proteins have been accepted as being critical both for the pore to form and in the regulation of its function (Halestrap and Richardson 2015, Richardson and Halestrap 2016). A previous, and a current hypothetical model for the structure of the pore is shown in Figure 1-6 (Karch and Molkentin 2014); the model has undergone, and continues to undergo revision as new discoveries are made and old findings reinterpreted.

1.3.1.1 Cyclophilin D

Halestrap & colleagues initially characterised the target for MPTP inhibition by CsA as a peptidyl-prolyl cis-trans isomerase and then identified this as cyclophilin D (Connern and Halestrap 1992, Connern and Halestrap 1996) which enhances the calcium sensitivity of the MPTP. Sanglifehrin A had also been previously observed to be a potent MPTP inhibitor; it was further found to act on cyclophilin D, albeit at a different site to CsA, confirming the role of cyclophilin D in the MPTP (Clarke, McStay et al. 2002). Cyclophilin D is not permanently present in the mitochondrial membranes; binding is dynamic, and is increased under conditions of oxidative stress. Binding of cyclophilin D induces the permeability transition.

This binding is dependent on three proteins in the inner mitochondrial membrane; the adenine nucleotide translocase (ANT), the inorganic phosphate carrier (P_iC), and the oligomycin sensitivity conferring protein (OSCP), which is a component of the F_1F_0- ATPase (Crompton, Virji et al. 1998, Woodfield, Ruck et al. 1998, Giorgio, Bisetto et al. 2009, Gutiérrez-Aguilar, Douglas et al. 2014). Both the P_iC and ANT display increased cyclophilin D binding under conditions of oxidative stress (McStay, Clarke et al. 2002), leading to pore formation.

Further, in mitochondria from CyP-D knockout mice, permeability transition is not sensitive to CsA and is also much less sensitive to increases in calcium concentration, thus demonstrating the vital role cyclophilin D plays in the formation of the MPTP (Baines, Kaiser et al. 2005, Basso, Fante et al. 2005). Nevertheless, in the presence of a suitably intense stimulus to pore formation, permeability transition is known to

occur in the absence of cyclophilin D (42) such that is not a necessary component of the MPTP.

1.3.1.2 Phosphate Carrier (PiC)

The inner mitochondrial membrane is impermeable under normal circumstances to small solutes. The phosphate carrier imports phosphate across the IMM which is required in order for oxidative phosphorylation to take place. Phosphate is thought to sensitise the MPTP to opening (Hunter, Haworth et al. 1976, Crompton, Ellinger et al. 1988) and so evidence began to accumulate implying this carrier as part of the MPTP (Varanyuwatana and Halestrap 2012). Further, in phosphate deplete environments, CsA is unable to inhibit the MPTP; implying an interaction between P_iC and CypD (Basso, Petronilli et al. 2008).

1.3.1.3 Adenine Nucleotide Translocase (ANT)

The adenine nucleotide translocator exchanges ATP for ADP across the inner mitochondrial membrane bidirectionally depending upon the electrochemical gradients; that is by the relative concentrations of the nucleotides and the mitochondrial membrane potential (Klingenberg 2008).

The ANT is also thought to have a significant role in MPTP opening. Regulators of apoptosis, including Bcl-2 are thought to mediate this effect via ANT binding and modulation of the opening likelihood of the MPTP (Halestrap and Brenner 2003). Given what has been shown about the criticality of cyclophilin D to MPTP opening, the demonstration of ANT binding cyclophilin D in a manner dependent upon cyclosporin A, and also proportionate to induced oxidative stress, serves to strengthen the argument that it too is a component of the MPTP, at least in some circumstances.

That caveat exists as doubts over the necessity of the ANT in the MPTP arose with the observation of MPTP opening in ANT1/2 knockout mice (Kokoszka, Waymire et al. 2004). However, the counterpoint was made by the same group that mitochondria from these animals were much less sensitive to calcium induced MPTP opening;

therefore the consensus position became that ANT presence was not essential for MPTP formation but served an amplifying role in its function, at least in the non-physiological setting of an isolated mitochondrial preparation.

1.3.1.4 Voltage Dependent Anion Channel (VDAC)

VDAC is a family of ion channels expressed on the outer mitochondrial membrane. , which behaves as a pore for small water soluble molecules, closing when the mitochondrial membrane potential rises above c. 40 mV. The role of VDAC in the MPTP is controversial. Antibody mediated blockage of the VDAC prevents mitochondrial permeability transition (Shimizu, Matsuoka et al. 2001); however the antibodies which were used in these studies may or may not be specific to membranes containing VDAC (Dermietzel, Hwang et al. 1994). Further biochemical evidence against its role is that knockouts of VDAC 1 have a normal permeability response to MPTP stimuli (Krauskopf, Eriksson et al. 2006); additionally, pulldown experiments demonstrated interactions of CypD with ANT and P_iC but not VDAC. This argues against the presence of VDAC in the pore, but is not conclusive (Woodfield, Ruck et al. 1998).

1.3.1.5 F_1F_0 ATPase

The ATP synthase has recently come to be thought to contribute to MPTP formation (Bernardi 2013, Bonora, Wieckowski et al. 2014). Indirect evidence from intact mitochondria found that the state of activity of the ATP synthase influenced the MPTPs ability to be opened by increased Ca^{2+}; others implied its presence electro-physiologically (Giorgio, von Stockum et al. 2013). Not all authors have been able to replicate these findings nor agree with the conclusion that the whole ATP synthase is necessarily present in the MPTP. Pinton, for instance, argues for a role of the c-subunit only; these subunits form the ring structure of ATP synthase in the inner mitochondrial membrane and so it is enticing to visualise how this ring might become a pore, in the absence of the other ATP synthase constituents (Bononi, De Marchi et al. 2013). Others have made an observation that silencing of the c- subunit causes a reduction in MPTP opening in a similar fashion to that seen with CsA (Alavian, Beutner et al. 2014).

1.3.2 Physiological Role of MPTP

Since its discovery, there has been much discussion about whether this pore has a physiological role or if it is purely a pathological phenomenon. Most experimental investigations have studied CypD, rather than the MPTP as a whole, and in particular as it relates to Ca^{2+} homeostasis and metabolic uncoupling.

Altschuld, in some of the earliest experiments, showed that treatment of cardiomyocytes with CsA as a pore inhibitor prevented mitochondria Ca^{2+} efflux (Altschuld, Hohl et al. 1992). Subsequently Elrod (Elrod, Wong et al. 2010) found that in a CypD mouse knockout model the mice became more sensitive to pressure-overload induced hypertrophic cardiomyopathy, associated with elevated mitochondrial matrix Ca^{2+}. The result of this is an increase in glucose utilization as a metabolic substrate rather than fatty acids; this limits the metabolic reserve in response to stress (Ong, Samangouei et al. 2015). Others have shown that CypD mediates Ca^{2+} transfer from the endoplasmic reticulum to mitochondria via an interaction with VDAC in cardiomyocytes (Paillard, Tubbs et al. 2013). It seems then that the MPTP may be important in the physiological regulation of Ca^{2+} uptake and efflux from the mitochondria; this role is much less well established than the role of the pore during I/R injury, and the significance of these observations is not yet clear.

1.3.3 Role of MPTP in I/R Injury

Even before CypD was identified as a component of the MPTP, CsA was known to have the ability to reduce hepatic injury in a model of I/R injury to the liver (Kurokawa, Kobayashi et al. 1992). In parallel, the MPTP was investigated as a mediator of acute myocardia I/R injury which showed MPTP opening was regulated by factors such as Ca^{2+}, inorganic P_i, oxidative stress, and purinergic nucleotides in studies which contributed to the structural model (Al-Nasser and Crompton 1986, Crompton, Ellinger et al. 1988, Ong, Samangouei et al. 2015). These factors are all known to change during acute I/R injury, and enhance the opening probability of the MPTP in this context. However, during the ischaemic phase, lactate accumulation results in strongly acidic conditions, with a pH less than 7.0, resulting in a strongly inhibitory effect upon the MPTP despite an environ which would otherwise favour its

opening, only becoming permeable early in reperfusion when the washout of lactic acid and with ATP availability reactivating NHE normalises pH (Griffiths and Halestrap 1995). Inhibition of MPTP opening is thus an attractive target for cardioprotection.

1.4 Cardioprotection

As discussed in Section 1.2, I/R injury involves a complex interplay of features and mechanisms interacting to produce injuries in differing modalities and at varying rates. However damage is done to the heart, it is important that it is minimised both in the adult and in the immature; as even in the developing heart after a certain stage, cardiomyocytes killed are not replaced by dividing progenitors (Yellon and Downey 2003). Figure 1-7 schematically shows the mechanisms that lead to injury and suggests interventions with which they may be targeted.

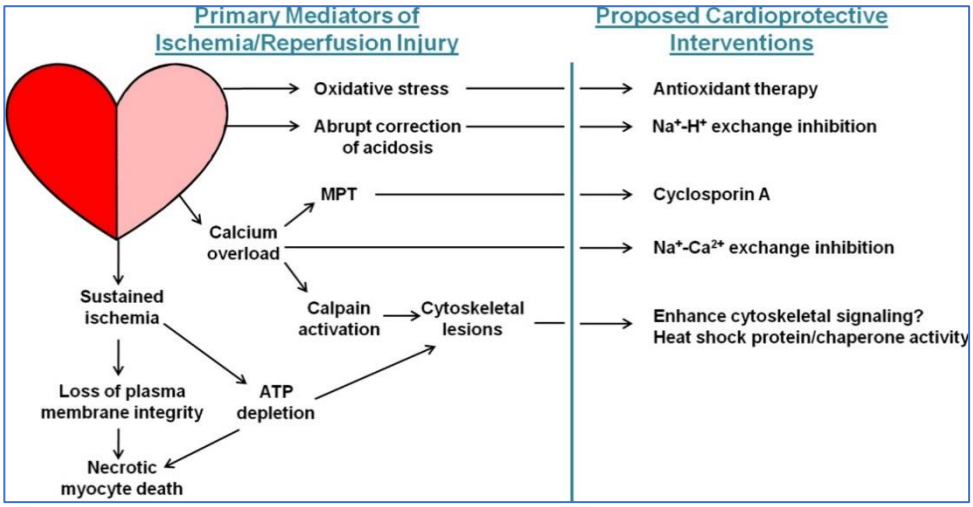


Figure 1-7 Mechanistic view of potential sites for cardioprotection(Perricone and Vander Heide 2014)

1.4.1 Intrinsic Cardioprotective Interventions

1.4.1.1 Ischaemic Preconditioning

Multiple strategies to protect the heart by non- pharmacological interventions have been described. Ischaemic preconditioning is perhaps the best known of these; it was hinted at clinically with the observation that the outcome for patients who described multiple episodes of *angina pectoris* prior to a myocardial infarction was improved compared to those who did not. However the key experimental study in 1986 showed that a dog's heart suffered a reduced size of infarct if the global ischaemic insult followed four 5 minute cycles of ischaemia (Murry, Jennings et al. 1986). This was then shown to operate by inhibition of MPTP opening in conditions where it would be expected to (Javadov, Clarke et al. 2003); the intermediate steps are not clear, although reduction in intracellular Ca^{2+} or in the generation of ROS were

proposed. Various signalling modalities were nevertheless known to be involved often including the phosphorylation of families of protein kinases; PKB/Akt, ERK 1 & 2 (Hausenloy, Tsang et al. 2005) as well as Protein Kinase C (Armstrong, Downey et al. 1994). A variant of this is ischaemic postconditioning, wherein the application of cycles of ischaemia to the heart following a significant ischaemic event- aortic cross clamping, or myocardial infarction, for instance- reduces the magnitude of injury sustained. The has obvious applicability for unanticipated ischaemia in the clinical setting, and is thought to have similar potency as ischaemic preconditioning (Zhao, Corvera et al. 2003).

1.4.1.2 Remote Ischaemic Preconditioning

An important mechanistic insight came from the observation that ischaemic preconditioning could be induced remotely, by shifting the initial ischaemic episodes away from the target organs to repeated cycles on a distant limb. A marked reduction in infarct size was again observed (Gho, Schoemaker et al. 1996), implying that there must be some form of signal imparted from the remote tissue to the heart.

The nature of this signal is not completely understood; the possibilities which have been postulated include a neurological signal from the ischaemic tissue, a release of humoral factors, or the triggering of some kind of systemic response. Nevertheless, similar mechanisms to those seen in direct ischaemic preconditioning have been observed involving activation of PKC (Wolfrum, Schneider et al. 2002, Hausenloy and Yellon 2008).

1.4.1.3 Temperature Preconditioning

Another notable protective strategy is temperature preconditioning. This refers to induced cycles of hypothermia prior to an ischaemic event reducing the injury that results. It also seems to reduce oxidative stress and so produce a relative reduction in MPTP opening, so reducing I/R injury. (Khaliulin, Clarke et al. 2007). Reducing temperature to around 26 °C seems to be the optimal strategy (Khaliulin, Halestrap et al. 2011).

Temperature protection confers protection by increasing myocardial cAMP and subsequent activation of Protein Kinase A (PKA) followed by activation of Protein

Kinase C (PKC) (Khaliulin, Parker et al. 2010). This sequence of events was confirmed by a finding from our group that the PKA inhibitor H-89 attenuated PKC activation following TP (Bain, Plater et al. 2007, Khaliulin, Parker et al. 2010) and led to the formulation of a new cardioprotective pharmacological strategy that involves cAMP/PKA/PKC signalling through consecutive administration of isoprenaline and adenosine.

Recent work in Bristol has shown that the normal adult rat heart can be effectively protected against I/R injury by transient and consecutive treatment with clinically relevant doses of isoprenaline & adenosine (Iso/Aden) (Khaliulin, Halestrap et al. 2014). This intervention has also been shown to protect hearts across a spectrum of models of pathology, when applied during hypothermic cardioplegic arrest (Khaliulin, Halestrap et al. 2011) and in rat models of heart failure and aging (Dudley, Suleiman et al. 2014, Khaliulin, Halestrap et al. 2014).

This intervention involves activation of Protein Kinase A (PKA) followed by activation of Protein Kinase C (PKC). A possible mechanism for this effect was noted with the observation that this treatment results in a decrease in glycogen content in the myocardium (Khaliulin, Parker et al. 2010, Khaliulin, Halestrap et al. 2014). Glycogen breakdown and subsequent accumulation of glucose-6-phosphate and low pH during ischemia triggers dissociation of mitochondrial hexokinase 2 (HKII) which can reduce mitochondrial permeability transition pore (MPTP) opening and cardiomyocyte death (Chiara, Castellaro et al. 2008, Pastorino and Hoek 2008, Pasdois, Parker et al. 2012).

1.4.2 Pharmacological Strategies

1.4.2.1 Protection by NHE inhibition

The intracellular fall in pH that accompanies ischaemia results in rapid increases in $[Na^+]_I$, then a subsequent rise in intracellular Ca^{2+}. Therefore, inhibition of the Na^+/H^+ exchanger may ameliorate that rise. Indeed this has been demonstrated experimentally (Liu, Cala et al. 2010) and interestingly, clinically, with a

demonstration of a fall from 20% to 16% 5 day risk of death in patients whom were receiving the NHE inhibitor cariporide in the EXPEDITION trial (Mentzer, Bartels et al. 2008). This trial however also showed an increased risk of mortality due to cerebrovascular events, and so the agent did not progress further. Nevertheless, this class of drug may prove useful for clinical cardioprotection.

1.4.2.2 Protection by NCX Inhibition

In a similar vein, it should hypothetically be possible to effect cardioprotection by directly inhibiting one of the sources of Ca^{2+} feeding the measured rise inside the cardiomyocyte. Inhibition of NCX is pharmacologically possible, with agents such as SN-6. Whilst the NCX may be inhibited, the agents are only effective at ameliorating the rise in Ca^{2+} if administered prior to the onset of ischaemia (Akabas 2004). They may potentially be useful therefore in circumstances such as surgery, where ischaemia may be planned and controlled, but of little use more broadly.

1.4.2.3 Protection with Antioxidants & MPTP inhibitors

Supplementation of the endogenous capacity to scavenge ROS should produce protection, as excess ROS generation is a key part of I/R injury. Experimental work in rats using the novel agent mitoQ initially appeared to reduce ROS production; however, in some others and in clinical work some cases of increased injury were observed (Gottwald, Duss et al. 2018, Zhou, Prather et al. 2018). Other work is ongoing in an attempt to discover an effective intervention- for instance, propofol, a $GABA_a$ agonist used widely and safely as a hypnotic agent, has been shown to inhibit the MPTP possibly because of its antioxidant activity (Javadov, Lim et al. 2000). Its cardioprotective efficacy during cardioplegic arrest has been confirmed in large animal studies(Lim, Halestrap et al. 2005). Its benefit in a clinical setting remains controversial (Plummer, Baos et al. 2014, Rogers, Bryan et al. 2015).

The most widely used agent to inhibit the MPTP remains Cyclosporin A, which has been used for this purpose since 1988 (Crompton, Ellinger et al. 1988) and exerts its effects through binding to cyclophilin D- section 1.3.1.1 (Halestrap and Davidson 1990). In experimental models, CsA produces protection against I/R injury in the isolated perfused whole heart model (Chen, Camara et al. 2007) and reduces necrotic cell death in an isolated cardiomyocyte model of simulated I/R injury (Nazareth, Yafei

et al. 1991). Given that CsA is acting as an MPTP inhibitor, it should be expected that its use during the phases of injury with maximal ROS production should be sufficient to produce protection. Indeed, perfusion with CsA is effective if given during reperfusion only, demonstrating that most MPTP opening occurs at this point (Hausenloy, Maddock et al. 2002, Argaud, Gateau-Roesch et al. 2005, Xie and Yu 2007). If perfusion with CsA is delayed, past around 15 minutes into ischaemia, no protective effect is seen, implying that the early reperfusion period is when MPTP opening occurs and determines the degree of injury (Hausenloy, Duchen et al. 2003). Other agents which inhibit the MPTP directly via CypD also have a protective effect, such as Sanglifehrin A (Clarke, McStay et al. 2002). Despite these promising pre-clinical results, these agents have not shown success as cardioprotective therapies in clinical trials, possibly because of their lack of specificity as pure MPTP inhibitors (Ong, Samangouei et al. 2015).

1.5 β-adrenergic receptor and cAMP signalling in the heart

Physiological signalling through neuroendocrine means is a key effector of homeostasis and the stress response. Hormonal release of adrenaline from endocrine tissues, and release of other adrenergic agonists through the sympathetic nervous system regulates the response of the cardiovascular system to changes in the environment, and physiological stressors.

β-Adrenergic receptor (β-AR) signalling is particularly important during conditions of acute stress, in order to mediate components of this neurohumoral response. cAMP is the main second messenger of the β-adrenergic receptor pathway (shown schematically in Figure 1-8), which acts mainly by activation of the cAMP dependent protein kinase, Protein Kinase A. There are a family of β-Adrenergic receptors, conventionally comprising three subtypes- β_1, β_2, and β_3. The receptors are integral membrane proteins and are part of the superfamily whose characteristic feature is of seven-transmembrane spanning domains known as G-protein coupled receptors. These are a key group of signal transduction proteins broadly involved in a wide range of physiological processes from olfaction through to mood regulation through interaction with neurotransmitters; however, their cardiac function is primarily limited to transmission of autonomic signalling.

There are well-characterised endogenous ligands for each of these receptor subtypes, and these substances often have activity at multiple receptors. Adrenaline, for instance, has activity at each receptor subtype and on the α- adrenoreceptors. However, pharmacological agents exist for selective function at each of these receptor types and so have been used as probes for investigating the respective roles of each distinct receptor; isoprenaline has long been used as a selective β_1 agonist.

Both β_1 and β_2 receptors are present in the heart, with a predominance of β_1 receptors; roughly 75% vs 25% β_2 in the human ventricle (Brodde 1991). Both of these receptor subtypes couple with G proteins, which are signalling intermediaries with GTPase activity. G proteins, or guanine- nucleotide binding proteins, are key in the transduction of receptor signals from the extracellular to intracellular spaces; they

are in an active state when bound to GTP, and inactive with GDP bound. For the membrane- bound G- proteins, such as those that interact with the β-Adrenergic receptors, they consist of three subunits- G_α and a $G_{\beta\gamma}$ complex. They are therefore known as the 'heterotrimeric' type. Activation of the GPCR to which a G- protein is bound, causes disassociation of the inhibitory $G_{\beta\gamma}$ dimer allowing unimpeded activity of the G_α subunit. Variation in response to GPCRs occurs therefore through the precise identity and targeting of the G-protein subunits or by ligand- specific conformational changes in GPCR (Gether, Lin et al. 1995); once activated and disassociated in the active form, the G_α may move away from the membrane to its targets for activation. There are multiple subtypes of G_α which couple with the β-AR; however, the β_1 receptor couples mostly with the G_s family, whose main target is adenylate cyclase. This enzyme when activated by G_s catalyses the conversion of ATP to cyclic AMP, a key signalling intermediary. Thus, this whole signal transduction cascade has both specificity in response, through the balance of G protein subtypes associated with receptors, and multiple amplification steps allowing small numbers of interactions at the cell membrane to cause a large output of messaging molecules.

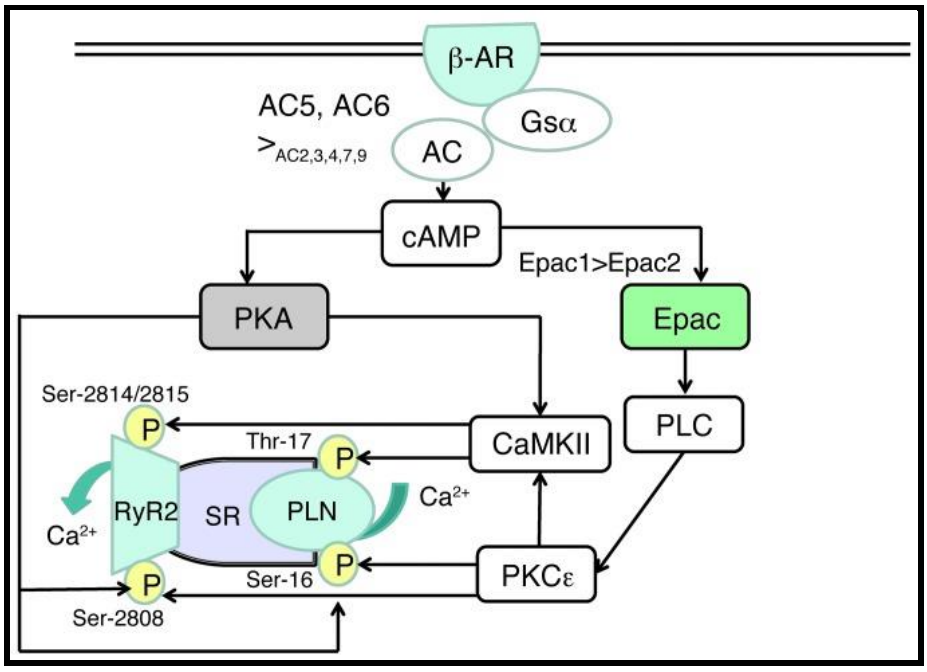


Figure 1-8 A schematic representation of β- adrenergic signalling with selected relevant downstream targetsB-AR= β-adrenoreceptor; Gsα= stimulatory G- protein; ACn= adenylate cyclase; cAMP= cyclic adenosine monophosphate; PKA= Protein Kinase A; Epac= Exchange protein activated by cAMP; PLC= phospholipase C; PKCε= Protein Kinase C epsilon; CaMKII= Ca2+ /calmodulin-dependent protein kinase II; PLN = phospholamban; SR= sarcoplasmic reticulum; RyR2= Ryanodine Receptor 2; P= phosphorylated residue From de Rooij., et al., 1998

1.5.1 Protein Kinase A

cAMP, as the molecule at a key nexus in the signalling cascades examined in this thesis, has multiple actions in the cardiac myocyte. Its canonical role was to cause activity of Protein Kinase A, PKA. This protein phosphorylates key proteins involved in EC coupling, including the L- type Ca^{2+} channel, ryanodine receptor (RyR2), troponin I (TnI) and phospholamban (PLN) (Bers 2002, Guellich, Mehel et al. 2014) (Figure 1-8). The key cardiac functions of β-AR are unsurprising given these targets; stimulation will cause a positive inotropic and chronotropic response in the heart, which follows from increased activity at RyR2 and the LTCC increasing calcium availability during systole (Guellich, Mehel et al. 2014).

The cAMP- dependent protein kinase, now known more commonly as Protein Kinase A, one the first protein kinases to be discovered (Walsh, Perkins et al. 1968). PKA is a tetrameric cytosolic protein consisting of two regulatory and two catalytic subunits (Corbin, Sugden et al. 1978). It is a commonly occurring signal transduction intermediary. In this form, the enzyme is inactive. When cAMP binds to this form, it causes a disassociation of the catalytic from regulatory domains; the resulting dimeric holoenzyme is then free to phosphorylate its targets.

In conditions where cAMP density is low, however, the inactive form of the enzyme predominates. So the overall activity of PKA is tightly controlled locally by the relative activities of adenylate cyclases, producing cAMP, and phosphodiesterases, degrading it. Regulation occurs then by the activity of these two influences in the vicinity of the PKA molecule, rather than merely by the abundance of PKA or cAMP *per se*.

Non- canonical influences upon the regulation of PKA have more recently become clear. For instance, in the cardiomyocyte under conditions of oxidative stress, activation of PKA independently of cAMP may occur bypassing the physiological regulatory mechanisms. This occurs by the reperfusion- induced production of H_2O_2 leading to the formation of disulphide bridges between RIα subunits of PKA. This leads to translocation of the kinase to the myofilaments and phosphorylation of its targets there, Troponin I and Myosin Binding Protein C leading to increased

contractility independent of the abundance of cAMP (Brennan, Bardswell et al. 2006).

Thus, activity of PKA is classically tightly regulated and controlled temporally by the activity of upstream receptors and associated signalling complexes, by regulatory influences upon the abundance of cAMP, but also by other influences such as redox signalling within a cardiomyocyte.

1.5.1.1 Spatial Targeting of PKA Signalling

Many proteins in different tissues contain motifs which may bind PKA and thus become phosphorylated. PKA is not free to diffuse passively around the cell, however; spatial targeting of PKA takes place through the association of the inactive PKA with A- kinase anchoring proteins (AKAP) confining the response to cAMP in a cell-type specific manner, allowing specificity in response (Dodge-Kafka, Soughayer et al. 2005) (Langeberg and Scott 2015). Without this function, the broad specificity of the catalytic subunits' phosphorylation site would not permit a response appropriate to the tissue. These were initially thought to be simple binding proteins, but are now recognized to be a very large family of multivalent enzyme scaffolding proteins that coordinate complex and highly organised intracellular events (Rubin 1994) and are even active and dynamic participants in signalling due to structural flexibility, and their ability to assemble a range of other regulatory proteins into a signalling nexus (Smith, Reichow et al. 2013).

Further regulation occurs by the regulatory subtype composition of PKA leading to the designation of PKA subtypes PKA-I and PKA-II (Smith and Scott 2018). Although these isoforms were historically thought to have a high degree of functional redundancy, differential compartmentalization through AKAPs is thought to provide distinct roles through spatial targeting.

1.5.1.2 PKA and the Mitochondria

This is of particular importance in considering the interaction of PKA with mitochondria; an AKAP tethers PKA to the outer mitochondrial membrane (OMM) (Figure 1-9) so localising a response which began at the cell membrane to an organelle some distance removed (Ould Amer and Hebert-Chatelain 2018) and increasing the efficiency and speed of the response.

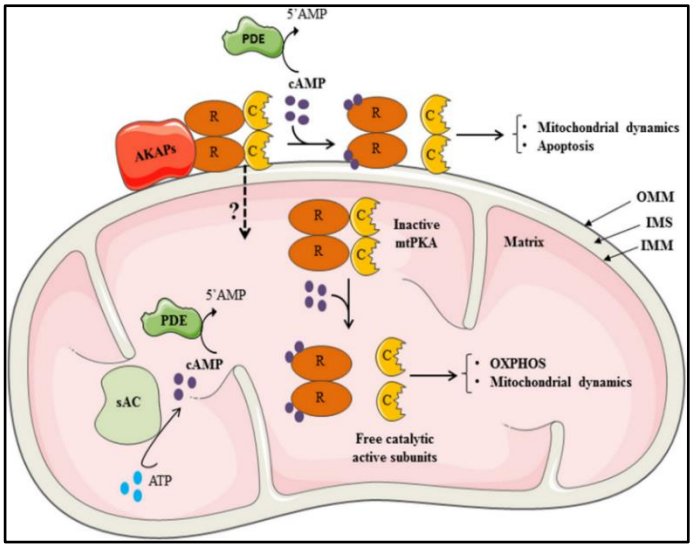


Figure 1-9 Association of PKA with MitochondriaFrom Ould Amer et al., 2018. PKA is tethered to the OMM by AKAPs which bind to the regulatory subunits of PKA (R).In the mitochondrial matrix, the soluble adenylyl cyclase (sAC) generates cAMP, whereas phosphodiesterase (PDE) degrades cAMP.. IMS: inter membrane space; IMM: inner mitochondrial membrane.

The tightly controlled spatial regulation of PKA activity mentioned above is also seen within the mitochondria, where two distinct subpopulations of cAMP are known to exist (Ould Amer and Hebert-Chatelain 2018). In the matrix, a soluble form of AC generates cAMP is thought to modulate oxidative phosphorylation through post-translational modification of components of complex IV (Acin-Perez, Salazar et al. 2009), whereas the transmembrane form of AC found at the OMM elevates cAMP there, associated with mitochondrial morphological changes, alteration to the mitochondrial membrane potential and seems to protect against apoptosis (Monterisi, Lobo et al. 2017) through phosphoinhibition of Bad. This is accomplished by PKA recruitment by AKAP121 (Affaitati, Cardone et al. 2003).

The precise nature of signalling at these sites is not solely a consequence of resulting PKA activity, either; AKAPs also recruit other populations of enzymes- non PKA

kinases- creating focal points for signalling pathways and their regulation (Dodge, Khouangsathiene et al. 2001, Feliciello, Gottesman et al. 2001, Dodge-Kafka and Kapiloff 2006).

Other than by interaction with mitochondrial membrane proteins, PKA has widespread influence on other aspects of mitochondrial function. Mitochondrial matrix PKA targets complexes I, IV, and V of the electron transport chain; in complex IV, for instance becomes phosphorylated by PKA during hypoxia at certain subunits decreasing overall complex IV activity (Prabu, Anandatheerthavarada et al. 2006). The net effects of these interactions are complex and incompletely characterised (Papa, Scacco et al. 2002) but mutations of these sites lead to significant developmental and neurological disorders, hinting at the key role PKA plays in ontogeny.

Collectively, it seems that mitochondrial metabolism is finely regulated by PKA activity, which is itself under the control of many convergent processes.

However, PKA is not the only effector of cAMP signalling. There are two targets which are as yet not well understood, the cyclic nucleotide gated ion channels and Popeye domain containing proteins, which are activated by cAMP (Simrick, Schindler et al. 2013, Brand, Poon et al. 2014), and a family of proteins known as EPAC which is a subject of active interest (Fujita, Umemura et al. 2016).

1.5.2 EPAC

1.5.2.1 Discovery

EPAC is a small family of signalling proteins discovered independently by two lines of investigation in 1998. de Rooij (de Rooij, Zwartkruis et al. 1998) and colleagues successfully identified a guanine- nucleotide exchange factor for Rap1. They were investigating stimuli that would activate the oncosuppressive properties of Rap1 (a small GTPase with sequence similarity to Ras), and discovered that activation of Rap1 occurs independently of activation of Protein Kinase A. This was achieved by

examination of cAMP- linked Rap1 activation in a cell line (CHO10248) with a mutant regulatory subunit of PKA which was essentially unresponsive to cAMP. Forskolin stimulation, alongside H89 induced PKA inhibition, of both these cells and a control cell line without the mutant PKA, showed equivalent Rap1 activation. Therefore, it was concluded that Rap1 was activated by a cAMP- stimulated factor independently of PKA. A database search was conducted for a protein with both GEF homology and cAMP binding sites. A sequence was identified by this search, and by an RT-PCR approach a strand of DNA encoding an 881 amino acid protein was isolated. They dubbed this protein EPAC- the Exchange Protein Directly activated by cAMP.

In parallel, another group (Kawasaki, Springett et al. 1998) were investigating the observation that several neuronal functions including neuroplasticity and neurotransmitter- initiated signalling were clearly involving cAMP but could not be convincingly linked to PKA. A search for guanine exchange factors with specificity for Rap proteins via a cloning approach resulted in the identification of a brain-enriched gene, CalDAG-GEF1 (Kawasaki, Springett et al. 1998). The product of this gene was found to have Rap1A specificity, bound both calcium and diacylglycerol, and had particularly enriched expression in axon-terminal regions of the brain. These features pointed strongly towards a signalling molecule, particularly one linked to the features of plasticity and neurotransmission.

1.5.2.2 EPAC Structure

Schematically, each of the two identified forms of EPAC are similar in their overall structure (Figure 1-10). They are made up of two functionally independent regions, a C- terminal catalytic region with guanine exchange activity, and an N- terminal region which is regulatory (Borland, Smith et al. 2009). EPAC then exists in two main conformational states in equilibrium with one another; a closed state, where the regulatory region sterically prevents access to the GEF site, and an open state where the catalytic site is freely available. In the presence of cAMP, the open state is thermodynamically favoured so catalysis is enhanced in high- cAMP states (Rehmann, Das et al. 2006, Rehmann, Arias-Palomo et al. 2008).

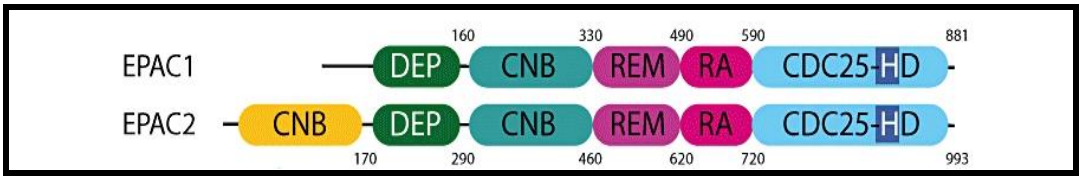


Figure 1-10. Schematic representation of the two described forms of Epac (Rehmann 2013)CNB= cyclic nucleotide binding domain; Dishevelled-Egl-10-Pleckstrin; REM= Ras exchange motif; RA= Ras association; CDC25-HD= CDC25 homology domain.

There are two main forms of EPAC, 1 & 2, and they have been best characterised in mouse and in human. EPAC 1 and 2 differ in the precise make-up of their constituent regulatory domains. In EPAC 1, this region consists of a DEP domain (after Dishevelled, Egl-10, and Pleckstrin; three initial proteins in which this domain was found) which is responsible for membrane localisation, and a single cyclic nucleotide monophosphate domain. By contrast, EPAC 2 retains the DEP domain but it is flanked on either side by cNMP domains. In both, however, the catalytic region is immediately preceded on its N-terminal side by a cNMP domain; the additional cNMP region of EPAC 2 does not seem to modify its capacity to be regulated by cAMP; indeed it has a relatively low affinity for cAMP of only 70 µM compared to 1 µM for the cNMP-B domain shared by both isoforms (de Rooij, Rehmann et al. 2000).

The EPAC 1 and 2 proteins, while broadly similar in their structure and *in vitro* biochemical activity, have differing physiological functions owing to distinct tissue distributions (Robichaux-III and Cheng 2018). In the mouse, *Rapgef3,* which encodes EPAC 1, is ubiquitously expressed. However, *Rapgef4*, which expresses EPAC 2, is more limited in distribution. Studies of mRNA abundance in mouse identify elevated levels of EPAC 1 in kidney, ovary, muscle, thyroid and brain tissues; EPAC 2 is mainly found in the CNS and adrenal, with very limited amounts detected in heart, small intestine and testis (Kawasaki, Springett et al. 1998).

Further, rodent studies suggest that EPAC 1 and 2 abundance is developmentally regulated. EPAC 2 seems to show more drastic changes through development in mice; but EPAC 1 mRNA expression does increase moderately after birth, reaching a maximum at 3 weeks post- natal in heart and some other tissues (Robichaux-III and Cheng 2018). However, in the brain, spinal cord and dorsal root ganglion tissues (in

rat) there is a complex developmental pattern of rising and falling levels of EPAC 1 & 2 at differing developmental stages (Murray and Shewan 2008).

1.5.3 EPAC function

Both EPAC 1 and 2 target the same immediate effectors; these are GTPases from the Ras superfamily known as Rap1 and 2. Despite this, both isoforms have distinct signalling roles and are not redundant due to their unique intracellular localisation as well as presence in signalosomes targeting particular partners. The EPAC proteins participate in a wide range of physiological functions summarised in Figure 1-11.

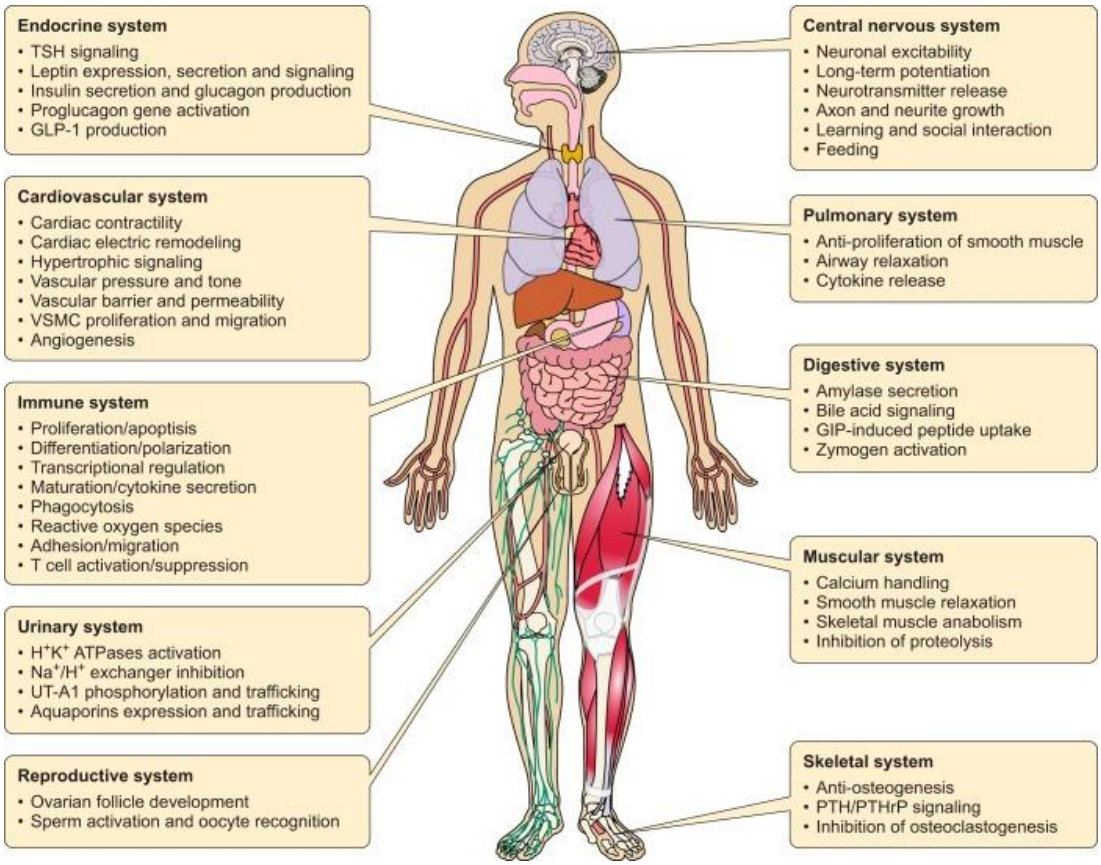


Figure 1-11 From Robichaux-III and Cheng 2018. A systems based summary of some the physiological functions EPAC proteins are known to be involved in.

1.5.3.1 EPAC1

EPAC 1 is known to exist at multiple locations within cells in a variety of tissues, and this localisation is dependent on the particular stage of the cell cycle in which a cell is examined. During interphase, it is mainly cytosolic in a perinuclear location as well as found in the mitochondria; however during mitosis it has been described as

translocating to the spindle and centrosome (Qiao, Mei et al. 2002). This tight association with translocation through cell division implies that the localization of EPAC 1 is strictly controlled and has functional consequences (Robichaux-III and Cheng 2018). cAMP, as well as being the activator of EPAC 1, is also responsible for influencing its location (Ponsioen, Gloerich et al. 2009). For instance, high concentrations of cAMP are associated with membrane localisation of EPAC 1 in a manner thought to depend upon a conformational change in the EPAC DEP domain contingent on cAMP binding (Consonni, Gloerich et al. 2012). This property allows signal transduction to occur in distinct compartments responsible for individual function roles of EPAC 1.

1.5.3.1.1 Functions of EPAC 1 at the plasma membrane

At the plasma membrane, EPAC 1 interacts with a family of scaffolding proteins known as ERM (ezrin-radixin-moesin) activated by PIP_2 binding and phosphorylation. This interaction between EPAC and ERM proteins occurs in a non- cAMP dependent manner; thus GPCR induced activation of ERM leads to localisation of EPAC at the plasma membrane (Gloerich, Ponsioen et al. 2010) promoting adhesion of the cell to the extracellular matrix in a manner important for cell spreading. This seems to occur downstream of Rap1 activation (Ross, Post et al. 2011).

ERM proteins are also capable of acting as AKAPs, through the ability of their helical domains to interact with PKA (Dransfield, Bradford et al. 1997). This provides a means for functional overlap at the plasma membrane between the roles of EPAC and PKA. EPAC 1 itself directly plays a role in the compartmentalisation of this signalling through interaction with AKAPs and PDEs; influencing both the temporal and spatial characteristics of cAMP signal transduction (Misra and Pizzo 2012).

This also results in the recruitment of EPAC 1 to a number of membrane receptors, channels, and associated proteins through the association of ERMs with these structures; significantly this includes the β-adrenoreceptors (McClatchey and Fehon 2009). As one example of the complex set of interactions EPAC is involved in at these receptors, this interaction provides the means by which EPAC 1 participates in β-AR

mediated cardiac hypertrophy through EPAC 1 mediated activation of H-Ras via Rap2B-PLC signalling causing remodelling of the cardiomyocyte by the expression of pro-hypertrophic genes. EPAC 1 also interacts with GRK2, a significant regulator of GPCR function. GRK2 directly phosphorylates EPAC1 in its DEP domain, inhibiting translocation of EPAC 1 to the plasma membrane (Singhmar, Huo et al. 2016). Thus from these few examples it is apparent that at the plasma membrane, EPAC 1 sits at the heart of a complex web of interacting regulators and effectors. This complexity means that the functional consequences of EPAC activation at the plasma membrane occurs in a tissue- and cell-type specific manner.

1.5.3.1.2 EPAC 1 at the nuclear envelope

The most prominent intracellular location for EPAC 1 is in the nuclear envelope (Dodge-Kafka, Soughayer et al. 2005). In rat neonatal ventriculocytes, the discovery of a complex coordinated through mAKAP containing EPAC 1, and PKA as well as PDE4D3 and ERK5 demonstrated one means of EPAC 1 targeting to this location. This also co-locates the two main effectors for cAMP as well as a temporal regulator into a signalling complex. A similar mAKAP & EPAC 1 containing signalosome has also been observed in cardiomyocytes, containing phospholipase C-ε, PKC-ε and protein kinase D (Zhang, Malik et al. 2013). This complex functions to regulate both nuclear PKD as well as the response to hypertrophic signalling. It has been previously noted that in this complex EPAC 1 selectively activates phospholipase C-ε through the activation of Rap2B (Schmidt, Evellin et al. 2001).

A non- mAKAP related nuclear membrane targeting mechanism for EPAC 1 also exists. Components of the nuclear pore complex, including Ran, the Ran binding protein 2 (RanBP2) and nucleoporins associate strongly with EPAC 1. This is especially true of the interaction between the RA domain of EPAC 1 and Ran-GTP; this is a necessary part of EPAC 1 activation of Rap1, which is its key signalling target, which suggests that nuclear localisation is a key part of EPAC 1's function (Liu, Takahashi et al. 2010).

1.5.3.1.3 EPAC 1 at the Cytoskeleton

A further functional role has been identified for EPAC signalling in cytoskeletal dynamics and organisation. EPAC 1 interacts directly with tubulin at the mitotic

spindle assembly where binding promotes microtubule formation (Mei and Cheng 2005). EPAC 1 also is known to associate with other microtubule binding proteins through its CNB domain; thus linking cAMP driven activation of EPAC to cytoskeleton dynamics. As interaction with microtubules directly inhibits EPAC 1 driven Rap 1 activity, and association with the light chains of the microtubule associated protein 1A (LC1 & LC2) enhances activation of Rap 1, there is dynamic regulation of Rap 1 activity based upon the relative abundance of these regulators (Borland, Gupta et al. 2006, Borland, Smith et al. 2009).

The activity of EPAC 1 at these locations causes an increase in the microtubular growth rate towards the cell periphery in a manner dependent on AKAP 9 for recruitment, which is also a requirement for the downstream functions of integrin mediated cell adhesion(Sehrawat, Cullere et al. 2008, Sehrawat, Ernandez et al. 2011).

Signalling via EPAC 1 is also required for hypoxia & ATP induced centrosome-nuclear separation; and so EPAC 1 is a vital component of the centrosome positioning mechanism under conditions of cellular stress. The significance of this finding is unclear, but it does point to the key role of EPAC 1 in the response to stressors. (Agircan, Schiebel et al. 2014).

A further key role for EPAC 1 in its association with the cytoskeleton is through involvement in mitochondrial dynamics and positioning. EPAC 1 does localise to the mitochondria via an N-terminal targeting sequence and this is known to be of importance in mitochondrial fission and fusion during VSMC proliferation after vascular injury (Wang, Robichaux et al. 2016).

Another important role for EPAC 1 at the mitochondria of particular importance for this thesis is the observation that EPA 1 activity triggered by mitochondrial cAMP inhibits Ca^{2+} entry, so stabilising the mitochondrial membrane potential and thereby inhibiting apoptosis in cardiomyocytes (Wang, Liu et al. 2016). However, these observations have not been universally accepted; another study demonstrated mitochondrial EPAC 1 activation conversely increased Ca^{2+} concentrations and led to cell death (Fazal, Laudette et al. 2017). The role of mitochondrial EPAC 1 then is

unclear; it is clearly of key importance in the study of unanswered questions around I/R injury. The heterogeneity of the response to its stimulation by cAMP in the context of tightly regulated signalosomes is a subject of ongoing work.

1.5.3.2 EPAC 2 functions

Whilst EPAC 1 has a very widespread distribution and interacts with a large number of signalling partners, the known functions of EPAC 2 are relatively circumscribed by comparison. This difference is in large part explained by differences in their sequence and structure. As discussed in Section 1.5.2.2, EPAC 2 possess an extra N-terminal CNB domain of very low cAMP affinity which is essential for clustering of EPAC 2 at signalosomes including the granule docking sites in β cells. This extra domain causes steric hinderance over the nuclear pore localisation sequence conserved in EPAC 1, and so EPAC 2 primarily localises to the plasma membrane (Robichaux-III and Cheng 2018). There are also sequence dissimilarities between the RA domains of EPAC 1 and 2. These differences allow EPAC 2 to interact with activated Ras, regulating the recruitment of EPAC 2 to the plasma membrane (Li, Asuri et al. 2006). This interaction is also required for EPAC 2 to activate Rap1.

Indeed, one of the main unique known functions for EPAC 2 is as a constituent of signalosomes at synaptic vesicles and at exocytosis of insulin- containing granules. However, there are no known specific function of EPAC 2 in the heart or wider cardiovascular system and it is not thought to be present at all in the rat heart, the animal model used throughout the experimental work of this thesis (Khaliulin, Bond et al. 2017).

1.5.3.3 EPAC Function in the Heart

EPAC functions as a cAMP sensor and is responsible for a number of cardiac functions both acutely, and over longer periods in response to adrenergic stimulation classically ascribed to PKA activity (Schmidt, Dekker et al. 2013). One such function is control of intracellular calcium concentration in response to extrinsic adrenergic stimulation. Via the PKC epsilon isoform and CaMKII, Epac phosphorylates the

ryanodine receptor 2 and phospholamban (Oestreich, Wang et al. 2007, Oestreich, Malik et al. 2009), causing increased release of calcium from the sarcoplasmic reticulum. Although PKA also causes increased phosphorylation of these targets, it is thought although not proven that Epac will phosphorylate them at different sites thus providing an avenue for synergistic function. Further, Epac agonists may cause increased spontaneous oscillation in intracellular calcium; this is not observed with activation of protein kinase A and so is a potential mechanism by which Epac may radically alter the process of EC coupling (Hothi, Gurung et al. 2008). This is further exaggerated through a PLC mediated phosphorylation of Troponin I and Myosin Binding Protein C (Cazorla, Lucas et al. 2009); this enhances myofilament sensitivity to calcium so providing a mechanism by which Epac regulates cardiac myofilament function differentially to PKA.

Epac will alter cardiac functions over the longer term by influences upon gene expression and cardiac structure. Epac increases calmodulin expression (Ruiz-Hurtado, Domínguez-Rodríguez et al. 2012); a key function of calmodulin is to regulate CaMKII, so this mechanism provides a longer term pathway to maintaining CaMKII activity and ultimately increased inotropic cardiac pump function. It has also been found that Epac stimulation in rat cardiomyocytes induces components of gap junction- related elements such as connexin- family proteins as well as N- cadherin, as well as being associated with new gap junction formation (Somekawa, Fukuhara et al. 2005). This circumstantially implies a role for Epac in cardiac remodelling during heart failure, with the concomitant increase in β-adrenergic signalling that is observed in chronic disease. Further, Epac is a known regulator of apoptosis through the PKB/Akt pathways although Epac activity is not sufficient to cause cell death in the absence of other signalling input (Kwak, Park et al. 2008). Relatively little is known about the role of Epac in the regulation of cardiac function in health or in disease with nothing known about the role of Epac during postnatal cardiac development.

1.5.4 cAMP and cardioprotection

Section 1.4 discussed various strategies for cardioprotection of the heart. There is considerable debate on the final common pathways by which the various

conditioning treatments exert their effect. It was observed that preconditioning attenuates cAMP levels during ischaemic injuries in the rat and rabbit myocardium (Lochner, Genade et al. 1999), and preconditioning also shows multiple cyclic increases in cAMP and matched falls in phosphodiesterase activity for cycles of ischaemic preconditioning (Lochner, Genade et al. 1998). Thus signalling through the cAMP second messenger axis is important in cardioprotection.

Section 1.4.1.3 discusses a pharmacological strategy which mimics temperature preconditioning by stimulation of PKA and PKC (Khaliulin, Parker et al. 2010). This method reduces Ca^{2+} induced mitochondrial swelling and this effect is accompanied by a reduction in oxidative stress during ischaemia.

This cardioprotective intervention is initiated by an increase in cAMP which has already been implicated in cardioprotection (Asimakis, Inners-McBride et al. 1994, Lochner, Genade et al. 1999) in mature hearts. However, the downstream effector mechanism of cAMP/PKA-induced protection has not been fully elucidated. One possibility is glycogen depletion, which results in reduced accumulation of lactate and H+ and hence smaller compensatory increases in intracellular Na^{+} and Ca^{2+} (Cross, Opie et al. 1996). An important advancement in the field of cAMP research and in its involvement in cardioprotection has been the synthesis of cAMP analogues which allow selective experimentation on components of the downstream signalling pathways.

1.5.5 The role of cAMP analogues

The availability of cell permeable cAMP analogues (Table 1-1) that are selective activators and specific inhibitors of PKA and Epac, has helped in elucidating the cardioprotective role of cAMP/PKA/Epac signalling pathways (Dudley, Suleiman et al. 2014). They are key agents in selectively dissecting out the respective signalling roles of PKA and EPAC and so are used as probes throughout this thesis. A wide variety- over 100- substituted cAMP analogues have been described (Schwede, Bertinetti et al. 2015). These agents have been widely used in our laboratory and by others as probes to investigate cardioprotection in a range of experimental models and

allowed selection of appropriate concentrations for use in the experimental chapters of this thesis. Below is a brief description of previous work using these agents which has allowed for their validation. Comparison is made with an Isoprenaline concentration of 5 nM, which has been used in prior studies of cardioprotection in our group.

cAMP Analogue	8-Br-cAMP-AM (**8-Br**)	6-Bnz-cAMP-AM (**6-Bnz**)	8-CPT-2′-O-Me-cAMP-AM (**CPT**)
Function	Activator of both Epac and PKA	PKA activator	Epac Activator
Concentration used in this work	5 μM	10 μM	10 μM
Rationale for Use	• Equipotent Comparator to Isoprenaline • Bypasses function of receptor complexes	• Equipotent comparator to Isoprenaline • Selective PKA agonist • Discriminator against EPAC function	• Equipotent comparator to Isoprenaline • Selective EPAC agonist • Discriminator against PKA function

Table 1-1 List of cAMP analogues used in our studies and in published work.

The least selective, and most broadly understood of these compounds is 8-Br-cAMP which is a widely used compound. (Poppe, Rybalkin et al. 2008). This is known to be a compound with good selectivity and affinity as a non-selective agonist of both PKA and EPAC (Ogreid, Ekanger et al. 1989, Christensen, Selheim et al. 2003). However, this agent is relatively cell- impermeable. A modification of this compound is the addition of an acetoxymethyl group which masks the charged polar phosphate; the effect of this is to increase cell permeability. Intracellularly, this is cleaved by esterases and so releases the original 8-Br-cAMP compound. This allows the study of the intracellular effects of this compound at a much lower applied dose than without the modification (Kruppa, Keely et al. 1997).

6-Bnz is a site-selective activator of PKA which does not function as an EPAC agonist. It also does not function as a competitive antagonist of EPAC, and so it is suitable to be used as an EPAC negative control. It has been widely used for selective

investigation of PKA in a number of settings and tissue types, from hepatocytes and haematopoietic systems through to cardiomyocytes. Similarly, an acetoxymethyl modification (-AM) exists which significantly increases membrane permeability and is again cleaved by intracellular esterases (Leech, Dzhura et al. 2010).

8-CPT-2'-O-Me-cAMP is a specific EPAC activator which does not activate Protein Kinase A. The structural modification here methylates the free 2'-ribose hydroxyl group in cyclic AMP which is essential for stimulation of PKA. This makes CPT an extremely poor agonist of PKA and so allows specific discrimination of functions particular to EPAC (Enserink, Christensen et al. 2002).

In our laboratory, the effect of cAMP analogues was assessed by measuring downstream targets in H9C2 cells. Our group has used these cells to asses Epac activity by measuring the expression of the gene c-fos (Chen, Koh et al. 2004). In preliminary work (n=3) we found that activation of Epac alone (10 µM CPT) or simultaneous activation of Epac and PKA (5 µM 8-Br) resulted in a marked and similar increase in c-fos expression (unpublished data). This provides an approximate dose relationship for equipotency of effect between 8-Br and CPT; thus in this thesis for the comparative work involving CPT, a concentration of 10 µM was used throughout.

Similarly, Ser-157 phosphorylation on the PKA substrate VASP was used to compare inducible PKA activation by 8-Br and 6-Bnz (Khaliulin, Bond et al. 2017). In these experiments, equivalency of effect upon VASP was found with 8- Br as 10 µM 6- Bnz. Therefore, at these dose the stimulatory effect upon PKA seemed equivalent, and so again for the comparative aspects of this thesis a 6-Bnz dose of 10 µM was used throughout.

The cardioprotective involvement of cAMP/PKA/Epac signalling pathways was determined using cAMP analogues in Langendorff-perfused rat hearts were subjected to 30 min global normothermic ischaemia (37ºC) and 2 h reperfusion. Activators (5 µM 8-Br, 10µM 6-Bnz & 10µM CPT) were added to the perfusate for 5 minutes during the stabilisation period and were washed out for 5 min before switching to global ischaemia. Figure 1-12 shows the acute effect of PKA and Epac activators on cardiac pump function (product of heart rate and developed pressure, RPP (Khaliulin, Halestrap et al. 2014)). All activators (alone or in combination) produced an inotropic effect which was highest when both PKA and Epac are activated simultaneously (8-Br or CPT+ 6-Bnz) and consistent with outcome associated with acute β-AR stimulation (10nM Iso). Interestingly, using inhibitors of PKA (5 µM H-89) or Epac (1 µM ESI-09) reduced but did not completely prevent the response to 8-Br.

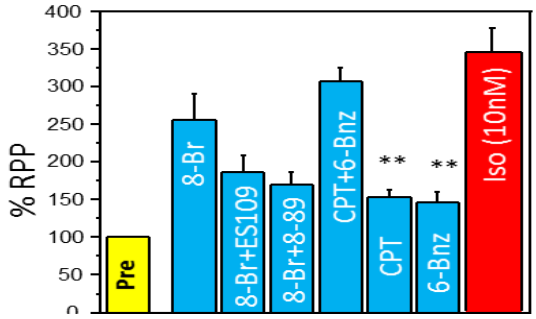


Figure 1-12 cAMP analogues are cardioprotective

*The data are presented as percentage change in RPP compared to pre-drug levels (taken as 100%). Mean±SE (n=5-9/group). **vs. 8-Br and CPT+6-Bnz. Peak %RPP levels in all interventions were higher (p<0.05) than pre-treatment. No difference between Isoproterenol (Iso) & activation by 8-Br or CPT+6-Bnz.*

Figure 1-13 shows the effect of cAMP analogues on infarct size following I/R. In **A**, hearts perfused with 8-Br (activator of both PKA & Epac) significantly reduced infarct size. However, the presence of inhibitors of either PKA (H-89) or Epac (ESI-09) blocked the cardioprotective effect of 8-Br. **B** shows that similar cardiopriection can be obtained by silultaneously adding two activators of both PKA (6-Bnz) and Epac (CPT). However, activation of Epac alone (CPT) did not show any protection whilst activation of PKA alone showed a small insignifcant reduction in infarct size. The effect of the latter was assocaited with a small but signifcant reduction in cardiac

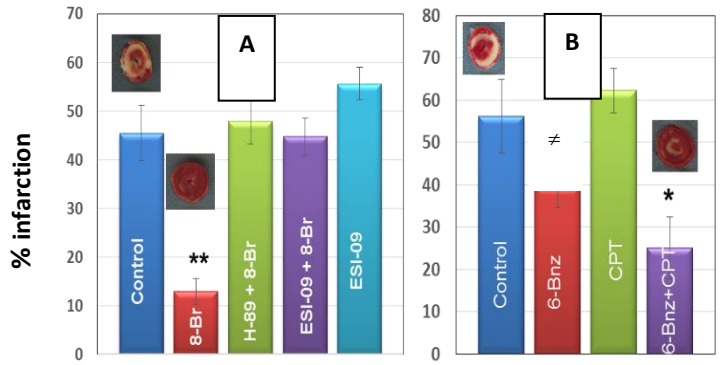


*Figure 1-13 The effect of cAMP analogues on infarct size in adult hearts exposed to I/R injuryThe addition of 5 µM 8-Br (an activator of both PKA & Epac) for 5 minutes prior to ischaemia conferred significant protection against I/R as shown by a significant reduction in % infarction. This effect was blocked by either ESI-09 or H-89. Panel B shows that simultaneous addition of 6-Bnz and CPT (activators of PKA & Epac) produced similar response to 8-Br. Insets are representative images showing infarction (white area) for selected interventions. Mean±SE (n=5-7/group).*vs. control & CPT. **vs. all other values. #vs. CPT. 6-Bnz showed a strong trend vs. control & 6-Bnz+CPT, but was not statistically significant. From Khaliluin.et al., 2017*

enzymes release during reperfusion as well as improved cardiac function (data not shown). It is worth pointing out that for all interventions, the extent of infarction correlated with functional recovery and cardiac enzyme release.

Main findings and implications: Our data using cAMP analogues suggest that cAMP signalling pathways induce a strong cardioprotective effect independently of β-adrenergic stimulation and that simultaneous activation of both PKA and Epac is required for optimal cardioprotection. These results are consistent with others showing lack of cardioprotection by Epac activation alone (Duquesnes, Derangeon et al. 2010) and with data demonstrating cooperative action of Epac and PKA in cardiomyocytes (Somekawa, Fukuhara et al. 2005). Finally, our published work and that of others implicate PKC in mediating cardioprotection induced by simultaneous activation of PKA and Epac.

1.6 Cardiac Development

The neonatal heart in a human is not a small version of the adult organ. It differs profoundly in both anatomical and pathophysiological domains. Therefore, it is not surprising that a heart at various stages of development will react differently to injury than a fully mature one in an adult cardiovascular system. In the UK each year, 9 in every 1000 children are born with congenital heart defects, of whom a significant proportion will require surgery. This surgery is often repeated, requiring multiple instances of anaesthesia, cardiopulmonary bypass and intensive care; each of these episodes causes injury to the heart and so finding methods to ameliorate this injury is important. These factors lead to a prevalence of over 200,000 people in the United Kingdom living with congenital heart disease. An understanding of quite how the young heart differs from the mature is then vital to developing protective strategies.

1.6.1 Anatomical development

There is rapid development of the post- natal heart as it matures towards the adult phenotype at a gross structural and ultrastructural level. During the first 11 post-natal days in rat, the heart weight increases more than 5-fold (Johnson and Brown-Borg 2006). After 4 days growth is hypertrophic (Anversa, Olivetti et al. 1980) rather than hyperplastic which ceases around the fourth post-natal day (Bicknell, Coxon et al. 2004); this is caused by cell cycle arrest of cardiomyocytes in G1 phase, possibly a result of regulation of β- adrenergic receptors during neonatal cardiomyocyte proliferation & growth (Tseng, Kopel et al. 2001). The overall physical characteristics of the neonatal organ differ from the adult as well, having significantly lower compliance; this may be a consequence of changes in composition. The neonatal heart has proportionately higher amounts of water, and proteins which do not contribute to contraction (Marijianowski, van der Loos et al. 1994, Pelouch, Kolar et al. 1997). The consequence of this is that the developing heart is less able to vary cardiac output by alteration of stroke volume and is more dependent upon heart rate for modification of output.

The neonatal heart is composed of a higher proportion of non-contractile protein, therefore it is less compliant than the adult (Marijianowski, van der Loos et al. 1994, Pelouch, Kolar et al. 1997). This has important consequences for how the developing heart can respond to external signals requiring an increase in cardiac work; with reduced compliance a developing heart cannot alter its stroke volume, and so is solely dependent on alterations in cardiac rate to increase or decrease output.

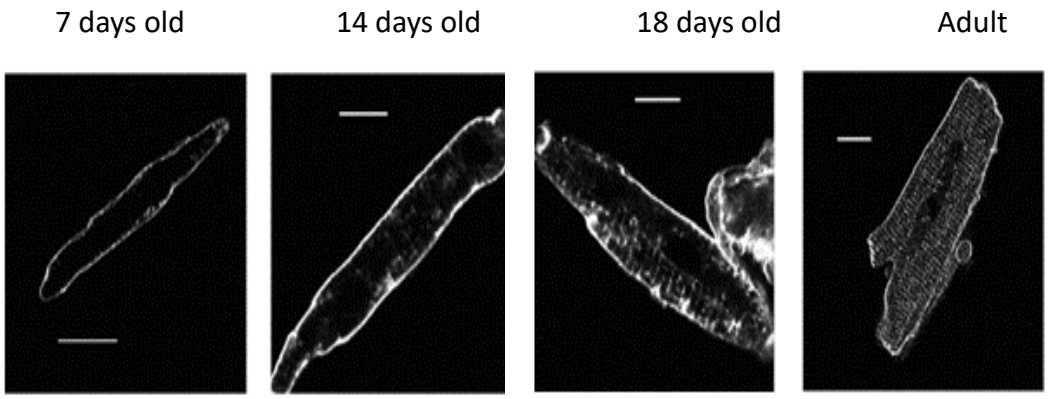


Figure 1-14 T-tubules from membrane-selective fluorescent dye di-8-ANEPPS-loaded rat ventricular myocytesFrom Seki. et al 2003

The morphology of cardiomyocytes changes dramatically during postnatal development: at birth cells are mononucleate, but after 14 days most cells become binucleate (Mayhew, Pharaoh et al. 1997). Soon after birth, the cardiomyocytes stop proliferating and instead they grow in size, and develop the rod-shaped striated morphology characteristic of adult cardiomyocytes (Figure 1-14)(Seki, Nagashima et al. 2003). At an ultrastructural level, from the first postnatal day until the seventh, neonatal cardiomyocytes display disorganised myofibrils primarily close to the cell periphery- an adaptation for efficient function given dependence on extracellular Ca^{2+} (Vornanen 1996). The intracellular Ca^{2+} stores are not fully developed, with little to no development of the t-tubule system or sarcoplasmic reticulum and so immature myocardium requires a higher extracellular Ca^{2+} concentration to optimise contractility (Jarmakani, Nakanishi et al. 1982).

1.6.2 Physiological Changes through Development

1.6.2.1 Calcium Handling

Cardiac pump function is well known to increase with post-natal age (Figure 1-15), but there are also corresponding changes in metabolic activity including glycogen storage and utilisation. The respiratory capacity of mitochondria & cytochrome levels increase postnatally (Marin-Garcia, Ananthakrishnan et al. 1997) whilst the sensitivity of the mitochondrial permeability transition pore to Ca^{2+} decreases with age (Balaska, Halestrap et al. 2005).

In addition, ionic homeostasis, excitation-contraction coupling, and its regulation, vary with age (Artman, Henry et al. 2000, Liu, Yasui et al. 2002, Tohse, Seki et al. 2004). For example there are qualitative and quantitative differences between Ca^{2+}-transients in cardiomyocytes from different age groups so that the rate of rise and decline are slower in younger animals compared to adult. There are also differences in the effects of inotropic and chronotropic agents during development in patients (Steinberg and Notterman 1994) and in experimental models (Driscoll 1987).

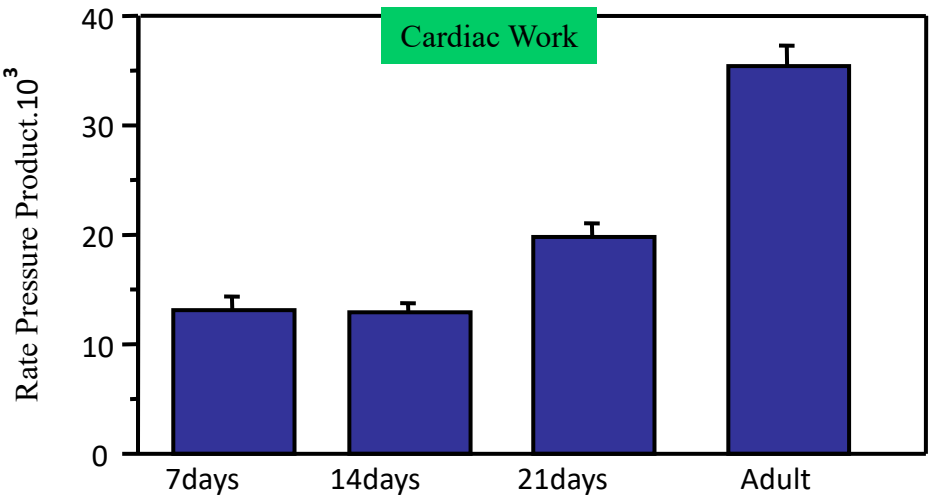


Figure 1-15 Increase in cardiac work, as estimated by rate pressure product, through key milestones during development of the rat heart(Suleiman group, unpublished data).

Developmental changes in Ca^{2+} cycling which contribute to these changes include altered abundance/ expression and activity of Ca^{2+} ATPase (SERCA), Na^{+}/Ca^{2+} exchanger (NCX) and L-type Ca^{2+} channels (Artman, Ichikawa et al. 1995, Vetter, Studer et al. 1995, Studer, Reinecke et al. 1997, Seki, Nagashima et al. 2003, Lin, Hung

et al. 2009, Wiegerinck, Cojoc et al. 2009)(Figure 1-16) as well as the development of SR and T-tubules, and the associated changes of protein location.

The sarcoplasmic reticulum and associated T-tubule network is underdeveloped for some time postnatally (Seki, Nagashima et al. 2003)(Figure 1-14). This is where the majority of proteins responsible for Ca^{2+} homeostasis are located (Brette and Orchard 2003), and so the mechanism of EC coupling in itself is changing markedly (Wibo, Bravo et al. 1991, Artman, Henry et al. 2000) through development.

In parallel, the location of the RyR within the developing cardiomyocyte, its density and role in the electrical properties of the cell changes throughout development (Snopko, Aromolaran et al. 2007) to support the shift towards a more SR dependent myocardium (Hamaguchi, Kawakami et al. 2013). The number of ryanodine binding sites doubles per unit mass tissue in the first month post-natal, alongside and in support of the developing SR (Wibo, Bravo et al. 1991).

The mechanisms for Ca^{2+} homeostasis are paradigmatically different in the immature heart, being much more dependent on extracellular than intracellular reserves, and so interventions targeting these Ca^{2+} stores may have differing consequences.

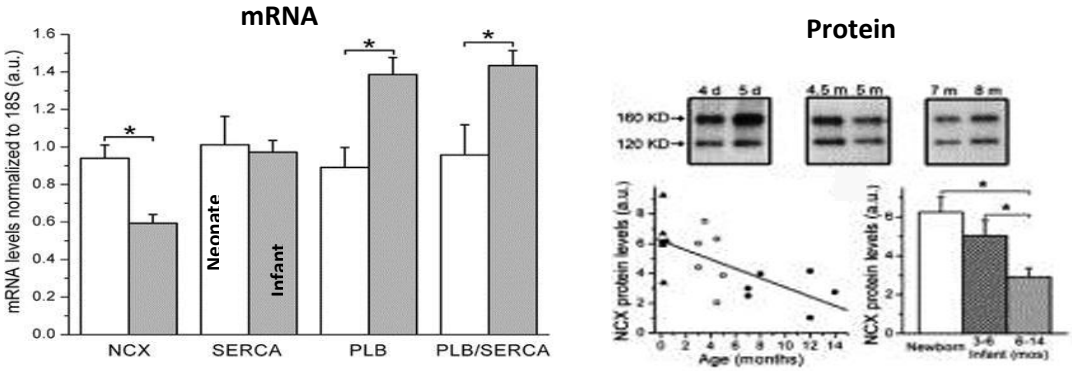


Figure 1-16 Ca2+ cycling proteins in neonates and infantsWiegerinck et al. 2009

1.6.2.2 Metabolic differences

As discussed in Section 1.1, the adult heart is dependent upon a variety of substrates to produce ATP, but the mainstay is the use of fatty acids. This is not thought to be the case in the neonate, which has a significantly higher dependence on glycogen than the adult, with a shift to fatty acid metabolism taking place as the heart matures. Accordingly, the reserves of glycogen which are high in the immediately postnatal heart drop between 7 and 14 days of age in the rat (Chen, De Diego et al. 2007) This may reflect the switch from a relatively oxygen deprived *in utero* environment to the oxygen rich *ex utero* world (Ascuitto and Ross-Ascuitto 1996). A consequence of this however is that the immature heart shows a reduced ability to produce ATP aerobically, with significantly higher fluxes of lactate and glucose in the immature heart. In parallel TCA cycle enzymatic activity increases with time post-natal (Veerkamp, Glatz et al. 1985).

Alongside this, there is a significant shift in the number, volume, and distribution of mitochondria through early maturation as might be expected to accompany the increased utilisation of oxidative phosphorylation. The number of mitochondria, as well as their density and size increase by a factor of 2 in the first 5 days post-natal in rats (Anversa, Olivetti et al. 1980), alongside development of the fine structure of the mitochondria themselves, with the immature mitochondria having relatively less prominent cristae, for instance, than the adult. Again, this suggests that the immature myocardium is significantly less dependent on oxidative phosphorylation as an energy source.

1.6.3 Age related differences in response to injury

Whether the resistance of immature mammalian hearts to the damaging effects of cardiac insults, such as hypoxia or ischaemia is higher or lower than adult remains controversial (Ostadal, Ostadalova et al. 1999), with some authors claiming that the immature heart has a greater resistance to ischaemia/ reperfusion injury and others the reverse. Most studies have tended to compare adult heart with a single selected developmental age group, rather than across the range of developmental stages.

1.6.3.1 *Evidence in favour of increased resistance in the immature heart*

The majority view is that the immature heart has significantly greater resistance (Doenst, Schlensak et al. 2003) to insults and tolerance of ischaemia than the adult heart (Riva and Hearse 1991, Ostadalova, Ostadal et al. 1998, Ostadal, Ostadalova et al. 1999, Ostadal, Ostadalova et al. 2014).

Work from King's College (Awad, Shattock et al. 1998) and from our laboratory (Modi and Suleiman 2004) demonstrated that recovery of developing rat heart following ischaemia and reperfusion changes during maturation and appears to follow a bell-shaped curve (Figure 1-17). Accordingly, 14-day old hearts show the highest resistance and adult hearts are the most vulnerable. Rabbit heart follows a similar profile (Figure 1-17, unpublished data), and our clinical research has shown age-related (e.g. neonate, infant, children and adults) differences in cardiac vulnerability to cardiac ischaemia/reperfusion in patients undergoing open heart surgery (Imura, Caputo et al. 2001, Modi, Imura et al. 2003).

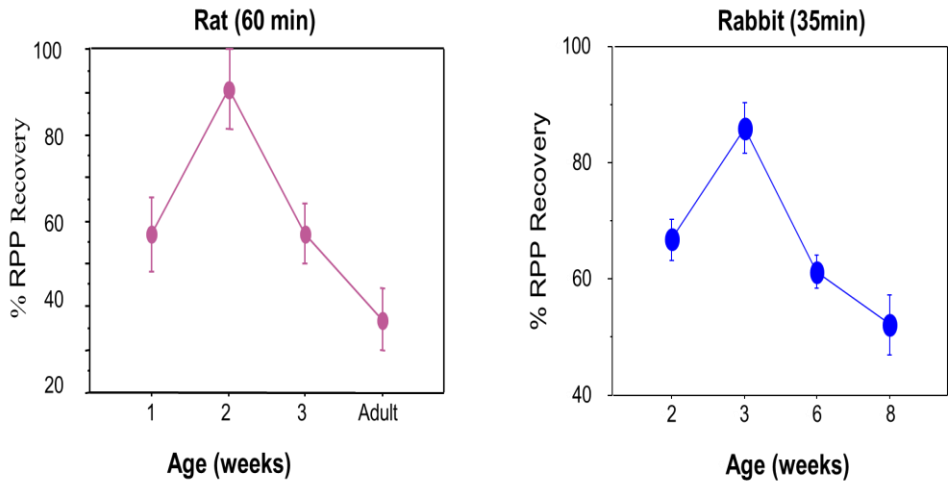


Figure 1-17 Vulnerability to cardiac insults changes during different stages of postnatal development Proportionate functional recovery compared to baseline after a period of global ischaemia (parentheses) in isolated perfused rat (left panel) and rabbit (right panel) heart.

The mechanisms underlying this profile of vulnerability to I/R during postnatal development are not currently known but this should be explicable from the anatomical, physiological, and metabolic changes discussed previously.

Ca^{2+} loading is key in mediating reperfusion injury and therefore developmental changes in Ca^{2+}-mobilisation could be responsible. In fact better preservation of Ca^{2+} handling has been reported in developing hearts following cardiac insults compared to adult hearts (Nakanishi, Young et al. 1984). Ca^{2+} levels during EC coupling are tightly controlled, involving mechanisms regulated in part by β-Adrenergic receptors. Manipulation of this system has also been linked to cardioprotection against I/R injury.

Further, the immature heart is thought to be better adapted to balance ATP production over hydrolysis especially in substrate deplete situations (Ostadal, Ostadalova et al. 2009); this may in part explain the observed pattern. These may be adaptations which reflect a relatively nutrient and oxygen poor *in utero* environment; this may involve reversion back to the fetal metabolism (Singer 2004).

1.6.3.2 Evidence against increased resistance in the immature heart

Opposing evidence has sought to demonstrate that the developing heart is in fact more susceptible to injury than the adult heart (Carr, VanderWerf et al. 1992, Portman, Standaert et al. 1996). One group studying the porcine myocardium found that in a model of ischaemia the adult took 50% longer before developing hypercontracture versus their developing comparator (Wittnich, Peniston et al. 1987). They explain this by suggesting that the developing heart both accumulates a larger acid load during ischaemia, but also has a lower capacity to buffer the H^+ (Wittnich, Su et al. 2006).

In observations on human patients undergoing cardiac surgery, others found the immature heart more vulnerable than the adult in measurements of postoperative troponin release in patients matched for cross-clamp time. However, these studies were very heterogenous in the comparisons made, from underlying demographics to the nature of the operations performed (Taggart, Hadjinikolas et al. 1996).

1.6.3.3 Resolution of Conflicting Evidence

At first glance these observations appear irreconcilable. However, those studies which demonstrate an increased tolerance to I/R injury in the adult only compare

against one particular age in development, versus every relevant developmental milestone. This is important as many groups have shown a rapid change in vulnerability throughout development, so by choosing only one time point as the comparator may miss the points of maximal resistance (Awad, Shattock et al. 1998, Modi and Suleiman 2004). Further, the conflicting experiments used multiple differing animal models and unsurprisingly these gave differing results; the timescales post- natal at which resistance may be maximal will change as each animal will develop at different rates with differing processes in that species also progressing at varying speeds; cross species comparisons are therefore fraught with difficulty. An approach based upon longitudinal study of the whole developmental period in a well characterised animal model would therefore be ideal, subject to the limitations of scope in a study and the need to minimise the numbers of animals studied.

1.7 Hypothesis

Transient combined PKA and EPAC stimulation prior to cardiac insults protects both neonatal and adult hearts and cardiomyocytes despite marked age-related differences in sensitivity to insults.

1.8 Objectives

The overall objectives of this thesis are therefore to define the age-related differences in vulnerability to I/R injury across a range of experimental models and to investigate how that vulnerability might be modulated using PKA and EPAC agonists. To achieve this objective, the following aims were addressed:

1. Construct a postnatal developmental profile of the expression and activation of proteins involved in cardiac cAMP/PKA/Epac signalling and Ca^{2+} cycling.
2. Investigate the effect of modulation of signalling along the β-adrenoreceptor axis on cardiac physiological status in the adult and immature heart and then compare that to following I/R.
3. Investigate the effect of PKA and/or Epac modulators on Ca^{2+} transients and contractility in cardiomyocytes under normal condition or during simulated ischaemia/reperfusion.

Since the immature heart is less vulnerable than the adult, it will be interesting to see whether it nonetheless may be protected by cAMP/PKA/Epac axis stimulation. If so, it would imply cardioprotection taking place through differing mechanisms than the intrinsic resistance. Further, the insights into the underlying biology may allow the suggestion of pharmacological cardioprotective intervention in adult heart to replicate the intrinsic resistance of the immature heart.

2 Materials & Methods

2.1 Materials

2.1.1 Buffers

2.1.1.1 Solutions used throughout the experimental work

The solution used to rinse tissue recovered from the heart to be used for biochemical experiments was a simple buffer based on a cardioplegia solution, composed of HEPES, glucose, KCL, $MgSO_4$ and $CaCl_2$ and adjusted to a physiological pH of 7.4.

Constituent	Mass/ Volume in 1L	Concentration
HEPES buffer(x10)	100 ml	20 mM
Glucose	2.7 g	15 mM
KCl	1.09 g	15 mM
$MgSO_4$	0.935 g	7.8 mM
$CaCl_2$	240 µl of 1M stock	1.2 mM

Table 2-1. Constituents of cardioplegia solution. Dissolved in ddH_2O & made up to 1l. pH measured and adjusted with HCl/ NaOH to 7.4.

Table 2-2 shows the constituents of the Krebs- Henseleit (KH) buffer used. This buffer is used for Langendorff perfusion of the whole heart and provides a relatively physiological ionic environment to support the automatic contraction of the heart in an *ex vivo* context, with glucose as a metabolic substrate. When used, this solution is also bubbled through with a gas mixture of 95% O_2/ 5% CO_2.

Constituent	Concentration(mM)
NaCl	120
Sodium Hydrogen Carbonate	25
Glucose	11
Potassium Hydrogen Phosphate	1.2
Magnesium Sulphate	1.2
Potassium Chloride	4.8
Calcium Chloride	1.2

Table 2-2. Components of the Krebs- Henseleit (KH) buffer used for whole heart perfusion prior to mitochondrial isolation

2.1.1.2 Cardiomyocyte Isolation

The main buffer used in cardiomyocyte isolation (Solution A, Table 2-3) is a physiological buffer adjusted to pH 7.25. Solution A is used to initially perfuse and equilibrate the heart. Two versions of A are produced, with 16 mM glucose and without. Glucose is omitted from the A used to produce solutions E, F, and G such that the cardiomyocyte experiments are performed without glucose. This was necessary in order to increase the injury incurred in our experimental model.

Constituent	Concentration (mM)
KCl	5
NaCl	137
$MgSO_4$	1.2
Na_2HPO4	1.2
HEPES	20
Glucose	16
$MgCl_2$	1.8
Sodium Pyruvate	5

Table 2-3. Constituents of Solution A. pH adjusted subsequently to 7.25 using NaOH, Glucose omitted from stock used to produce Solutions E, F, and G

Perfusion with solution A is then followed sequentially by the solutions shown in Table 2-4. Perfusion is first switched to B and then to C; D performs digestion, then following recovery of the cardiomyocytes Ca^{2+} is sequentially reintroduced by sequential addition of solutions E, F, and G.

Constituent	Concentration
Solution B	750 µM $CaCl_2$ & 16 mM Glucose
Solution C	90 µM EGTA & 16 mM Glucose
Solution D	10 mg/ml Type 2 Collagenase 1 mg/ml Protease
Solution E	150 µM $CaCl_2$
Solution F	0.5 mM $CaCl_2$
Solution G	1 mM $CaCl_2$

Table 2-4. Additives added to Solution A to form other Solutions used for cardiomyocyte isolation.

2.1.1.3 Mitochondrial Isolation Buffers

For isolation of mitochondria, a buffered sucrose solution is first made (Table 2-5) some of which is saved for use, and some of which is used as the basis of the other solutions used in the mitochondrial isolation experiments. A Final Wash (AFW) does not contain EGTA unlike the other mitochondrial isolation buffers and so is used as the last buffer in the isolation process before the addition of Ca^{2+} in a swelling assay.

Constituent	Concentration/ mM
Sucrose	300
Tris- HCl	10

Table 2-5. Components of Buffer A Final Wash for mitochondrial isolation. pH adjusted to 7.2.

Buffer AFW was then used to form two other buffers used for mitochondrial isolation, A & B (Table 2-6). Both of these buffers contain EGTA to chelate calcium; buffer B also contains additional BSA.

Constituent	Solution	Concentration
EGTA	Buffer A & B	1 mM
BSA	Buffer B only	5 mg/ml

Table 2-6. Components added to Buffer A Final Wash to form both Buffers A & B. pH maintained at 7.2.

Once isolated, the mitochondria are assessed for swelling as described in Section 2.5.3. This is performed in a swelling buffer used for de-energised mitochondria (Table 2-7). It contains a metabolic inhibitor, a calcium ionophore (A23187), rotenone (a complex I inhibitor), as well as antimycin A, a cytochrome c inhibitor.

Constituent	Concentration/ mM (except where noted)
KSCN	150
Tris	10
MOPS	20
NTA	2
A23187	2 µM
Rotenone	0.5 µM
Antimycin A	0.5 µM

Table 2-7. Components of mitochondrial swelling buffer (for de-energised mitochondria). pH adjusted to 7.2

2.1.2 Reagents

There are three synthetic forms of cAMP that were used throughout the experiments described in this thesis. They are shown in Table 2-8, with their full systematic name, abbreviated form used in the prose and their intended function.

cAMP Analogue	Abbreviation	Function
8'-bromoadenosine 3',5'- cyclic monophosphate- AM	8- Br	Non-selective agonist of PKA & EPAC
N6- benzoyladenosine- 3',5'- cyclic monophosphate- AM	6- Bnz	Selective PKA agonist
8- (4- Chlorophenylthio) adenosine- 3', 5'- cyclic monophosphate, acetoxymethyl ester	CPT	Selective EPAC agonist

Table 2-8. cAMP analogues used throughout this series of experiments. Supplied by Biolog, Bremen, Germany.

All other reagents used throughout the work described are listed in Table 2-9 along with their supplier.

Reagent	Supplier
A23187	Sigma Aldrich, Darmstadt, Germany
Antimycin A	Alfa Aesar, Ward Hill, Massachusetts, USA
Bovine Serum Albumin	Sigma Aldrich, Darmstadt, Germany
Bradford Reagent	Biorad, California, USA
Calcium Chloride, $CaCl_2$	Panreac, ITW Reagents, Barcelona, Spain
Cyclosporin A	Sigma Aldrich, Darmstadt, Germany
Dimethyl sulphoxide, DMSO	VWR International, Lutterworth, UK
ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, EGTA	Sigma Aldrich, Darmstadt, Germany
Fura-2-AM	Biotium, California, USA
Glucose	Sigma Aldrich, Darmstadt, Germany
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	VWR International, Lutterworth, UK
Hydrogen Peroxide, H_2O_2	Sigma Aldrich, Darmstadt, Germany
Isoprenaline hydrochloride	Sigma Aldrich, Darmstadt, Germany
Magnesium Chloride, $MgCl_2$	Fisher Scientific, Loughborough, UK
Magnesium Sulphate, $MgSO_4$	Fisher Scientific, Loughborough, UK
3-(N-morpholino)propanesulfonic acid, MOPS	Sigma Aldrich, Darmstadt, Germany
Nitrilotriacetic acid, NTA	Sigma Aldrich, Darmstadt, Germany
Nonidet P-40 detergent	Sigma Aldrich, Darmstadt, Germany
Sodium Cyanide, NaCN	Sigma Aldrich, Darmstadt, Germany
Sodium Hydrogen Carbonate, $NaHCO_3$	Sigma Aldrich, Darmstadt, Germany
Sodium Hydrogen Phosphate, NaH_2PO_4	Sigma Aldrich, Darmstadt, Germany
Pluronic Acid	Sigma Aldrich, Darmstadt, Germany
Potassium Chloride KCl	Fisher Scientific, Loughborough, UK
Potassium Hydrogen Phosphate KH_2PO_4	Sigma Aldrich, Darmstadt, Germany
Potassium Thiocyanate, KSCN	Sigma Aldrich, Darmstadt, Germany

Protease (Bacterial, Type XIV)	Sigma Aldrich, Darmstadt, Germany
Rotenone	Bio-Techne, Minneapolis, Minnesota, USA
Sodium Chloride, NaCl	VWR International, Radnor, Pennsylvania, USA
Sodium Cyanide, NaCN	BDH Laboratory Supplies, Poole, UK
Sodium Deoxycholate	Sigma Aldrich, Darmstadt, Germany
Sodium dodecylsulphate	Sigma Aldrich, Darmstadt, Germany
Sodium Hydrogen Carbonate, $NaHCO_3$	Fisher Scientific, Loughborough, UK
Sodium Pyruvate	VWR International, Radnor, Pennsylvania, USA
Sucrose	Sigma Aldrich, Darmstadt, Germany
Triphenyl tetrazolium chloride, TTC	
Tris	Sigma Aldrich, Darmstadt, Germany
Trypan Blue Dye	Sigma Aldrich, Darmstadt, Germany
Type 2 Collagenase	Worthington Biochemical, Lakewood, New Jersey, USA
Hydrochloric Acid, HCl	Sigma Aldrich, Darmstadt, Germany

Table 2-9. Other reagents used throughout these experiments, with systematic name where used elsewhere.

2.1.3 Animals

The use of the animals described in this thesis conformed to the Home Office regulations specified in the schedules of the Animals (Scientific Procedures) Act, 1986. All animal tissues were obtained from male Wistar rats (immature; from Animal Services Unit, University of Bristol) of 7- days, 14- days & 28- days post-natal as well as mature (>60- day old; Charles River Laboratories, UK) animals. Killing was performed humanely as described in Schedule 1 of this Act; this by stunning by cerebral concussion followed by cervical dislocation. Quantities of animals used for each particular experiment are detailed in the respective chapters.

2.2 Protein expression & quantification

2.2.1 Extraction of heart tissue

Hearts were extracted from Male Wistar rats of differing ages. Charles River Laboratories supplied adult rats of 250-300g in whole body weight, and 28- day old rats of between 100-125g in weight. The University of Bristol Animal Services Unit provided immature rats at 7- and 14- days postnatal age. In total, for these proteomic experiments 31 rats were used; adult (n=9), 28- day (n=9), 14- day (n=9), and n=4 for 7- day old.

These rats were rapidly killed by cervical dislocation. A midline incision exposed the thorax and then a bilateral thoracotomy incision was made to obtain access to the mediastinum. The heart and great vessels were manually lifted out of the chest and an incision made posteriorly towards the thoracic inlet in order to release them.

The hearts were then immersed in cardioplegia solution at 4°C and agitated in order to remove blood from the ventricles. They were then immediately immersed in liquid nitrogen, and stored at -80°C.

2.2.2 Homogenisation of heart tissue

In order to analyse the myocardial constituents, the hearts from 7- , 14- , and 28- day old male Wistar rats were homogenised. The most appropriate buffer for recovery of a representative sample protein content of the cardiomyocytes was chosen to be a RIPA buffer. Other buffers are used commonly in proteomic work; for instance urea based buffers have been commonly used in applications targeting membrane bound protein constituents. However as this series of experiments had no particular target, it was felt best to use an extraction buffer, which would provide as little bias to the resulting proteome as possible, shown in Table 2-10.

PBS Tablet x1
1% (v/v) Nonidet P-40 detergent
0.5% (w/v) Sodium deoxycholate
0.1% (w/v) Sodium dodecylsulphate

Table 2-10. Composition of RIPA buffer used for protein extraction from heart samples. Made up to 200ml with ddH2O and pH adjusted to 7.4 with concentrated HCl/NaOH solutions

A 10ml aliquot of RIPA buffer was taken, to which was added 1x tablet of phosphatase inhibitor and 1x tablet of protease inhibitor. The hearts were then thawed on ice. Small samples from the apex of each were cut from the heart of approximately equal size, and weighed. These samples were then placed in 2ml tubes containing 50x tungsten beads. To each of these samples was added 10 µl of RIPA buffer per mg of tissue.

A tissue homogeniser was then used to lyse the cells in the sample by oscillation. This was performed at 4°C; each sample was shaken in two bursts of 10 seconds, and then agitated on ice for 30 minutes. The samples were then centrifuged at 10000 g for 10 minutes at 4°C, which separated them into a pellet and supernatant. 300 µl of supernatant for each sample was recovered, split into 100 µl aliquots, snap frozen, and stored at -80° C. This supernatant should contain a majority of the protein content from the original tissue.

2.2.3 Protein Concentration Estimation & Normalisation

The Tandem Mass Tagging- Mass Spectrometry technique used subsequently for peptide detection and identification is performed at a standardised protein concentration. This was set at 2 mg/ml and so in order to achieve this, the concentrations of each sample had to be determined, and then diluted to produce concentrations at this standard. This was achieved spectrophotometrically by the Bradford method (Bradford 1976).

Using the Bradford reagent, a standard curve of light absorbance at 595 nm over a range of concentrations of BSA, a commonly used protein reference, was produced. The BSA absorbance was measured over a range of concentrations, repeated, and an average taken of the measurements.

The protein extract samples were then diluted 3- fold in order for their absorbances to appear within the range of this curve. The samples were therefore diluted 2:1 with ddH_20, and their concentrations calculated using the Bradford method at 595 nm. Two measurements were made for each sample, and the average absorbance calculated. The post- dilution concentrations were calculated with reference to the standard curve, and then corrected for the dilution. A dilution factor required to correct each sample to a normalised 2 mg/ml was calculated, and the corresponding quantity of ddH_2O added to each sample. These samples were then stored at -80°C ready for analysis.

2.2.4 Tandem Mass Tagging- Mass Spectrometry Quantitative Proteomics

The samples were then used for estimation of the whole myocardial proteome, with assistance with the technique from Dr. Kate Heesom of the University of Bristol Proteomics Facility at the Department of Biochemistry.

The TMT-MS approach involves enzymatic digestion of a sample breaking up proteins into constituent peptides (Thompson, Schafer et al. 2003).

The tandem mass tags function as labels by binding to the peptides produced by digestion of the sample. The mass tags bind specifically to a certain amine, and so each tag- amino acid pair has a defined mass. The samples, as well as a pooled sample created from all the individual samples, were labelled with these tags, then fractionated chromatographically. Each fraction was then analysed using an Orbitrap Fusion Tribrid mass spectrometer. During this analysis, the tags attached to each residue fragment, producing the reporter section of the tag which allows quantification of the peptide. Multiple peptides from each protein are produced, so

by taking the mean quantification from each of these peptides an accurate quantification is produced. Each experiment with the mass spectrometer could involve up to ten samples. Therefore, in order to make best use of the samples and resources, three runs were planned, consisting of 9 experimental samples and a pooled sample created from all of the samples.

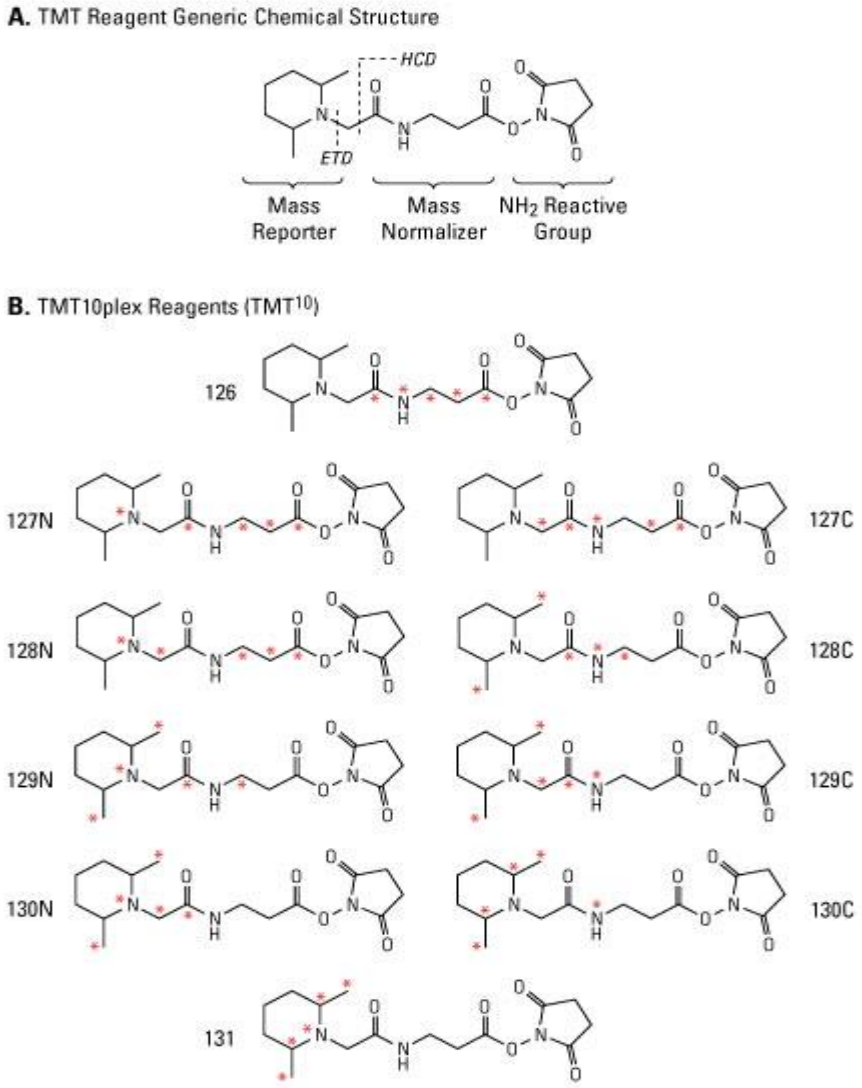


Figure 2-1. The structure of the amine-reactive TMT Reagents: A. Functional regions of the reagent structure. B. TMT10plex reagent structures with 13C and 15N heavy isotope positions (red asterisks). (From ThermoFisher https://www.thermofisher.com/order/catalog/product/90110)

The resulting raw data spectra were analysed using Proteome Discoverer version 2.1 (Thermo Fisher Scientific, Waltham, United States of America). This software produces a sequence for the peptides detected on the mass spectrum, a likely protein from which that peptide originates, and assigns a confidence level in each peptide/

protein detected as well as an abundance level of that protein relative to the pooled sample. Filters to exclude everything but the peptides with the highest (>95%) level of confidence were applied. Subsequent analysis of the data was performed using Microsoft Excel 2013 (Microsoft Corporation, Seattle, United States of America) and IBM SPSS Statistics 23 (IBM Corporation, Armonk, United States of America).

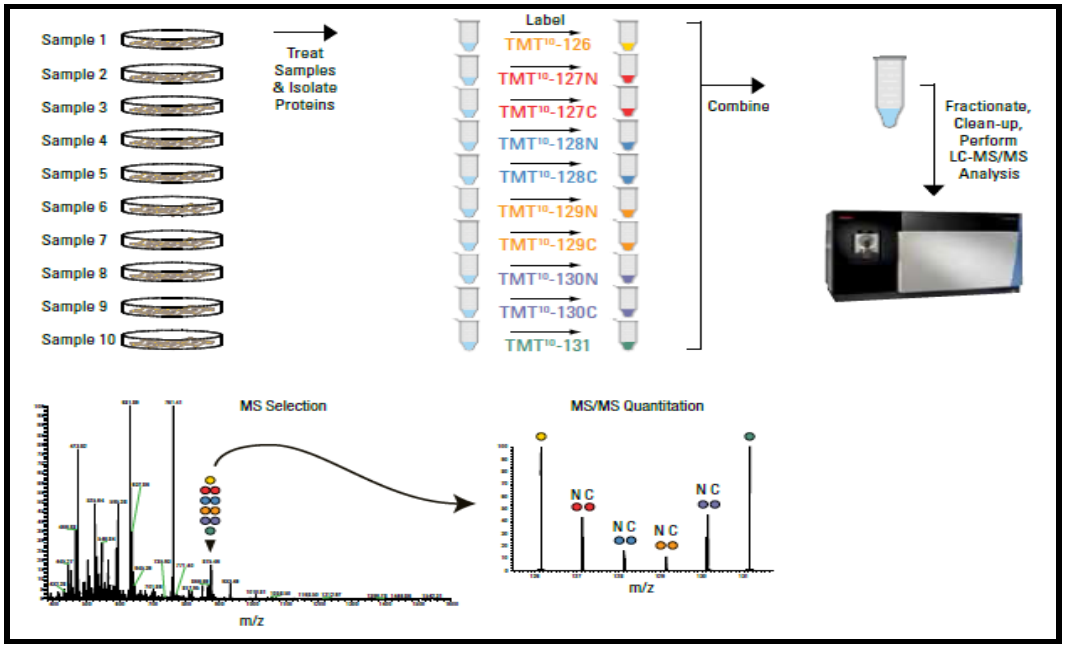


Figure 2-2. Scheme for MS experiments (adapted from ThermoFisher)Protein extracts are reduced, alkylated and digested overnight. Samples are labelled with the TMT Reagents and then mixed before sample fractionation. Labelled samples are analysed by high resolution Orbitrap LC-MS/MS before data analysis to identify peptides and quantify reporter ion relative abundance. (https://www.thermofisher.com/order/catalog/product/90110).

2.3 Ex vivo Whole Heart Langendorff Perfusion

2.3.1 Heart Extraction

Whole hearts were extracted from male Wistar rats of 14- days post-natal as well as adult animals. Animals were killed by cerebral concussion followed by cervical dislocation. For each animal, a sternotomy incision was made from the xiphisternum to sternal notch to expose the thorax, and then bilateral thoracotomy incisions with reflection of the ribs exposed the mediastinum and lungs. The heart was lifted gently out of the thorax and an incision made posteriorly along the vertebral column to cut through the descending aorta and release the heart. The heart was immediately placed in Krebs- Henseleit (Table 2-2) buffer chilled to 4° C and excess tissue around the atria and great vessels dissected away.

2.3.2 Perfusion equipment

Hearts were perfused using a Langendorff system supplied by AD Instruments (Sydney, Australia) (Figure 2-3, Figure 2-4). There are broadly two elements to this apparatus, a temperature control system and a perfusate delivery system. For temperature management, the apparatus consists of a pump, a water reservoir, heating element and integrated temperature sensor connected to a tubing system passing through the external walls of all the buffer reservoirs. Each reservoir has a jacket of two layers of glass with a void in between through which the warmed water passed to maintain each chamber at the desired temperature.

For these experiments on rats, the temperature of the system was maintained at 37° C which is physiological body temperature for these animals. The temperature of the perfusate at the cannula was measured regularly and the settings on the warming element adjusted to ensure the perfusate as it enters the heart was at the set temperature.

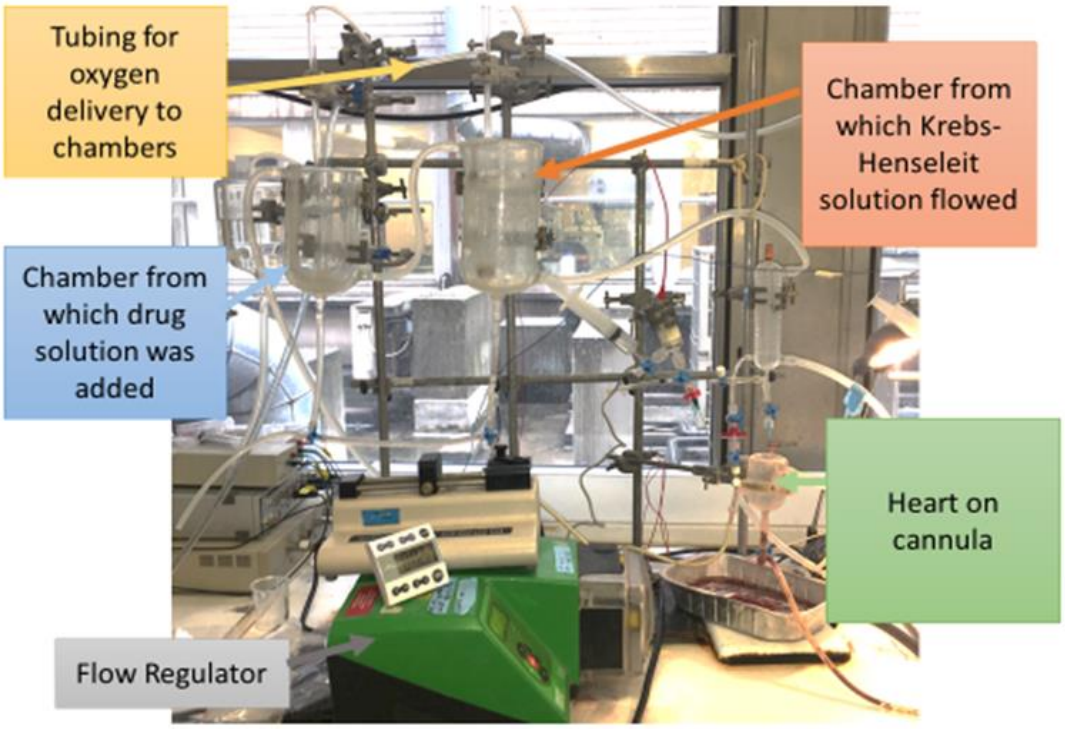


Figure 2-3 Example of apparatus used for Langendorff perfusion experimentsExperimental buffer placed in warmed reservoir, marked blue. 95% O_2/5% CO_2 bubbled through from cylinder supply, yellow. Flow controlled by roller pump. Image- Katie Hall.

Figure 2-4 Close up image of heart perfused on cannulaHere, 14- day old heart seen with 22G cannula entering aortic root and secured with Vicryl suture prior to clamp removal. Image- Katie Hall.

The equipment contains three reservoirs for perfusion buffers with needle valves. These valves allow a buffer within the reservoir to have an externally supplied gas passed through; this was used in these experiments to dissolve a gas consisting of 95% O_2/ 5% CO_2 through Krebs- Henseleit buffer. The purpose of this is to oxygenate the buffer so that in perfusing the heart it delivers oxygen; and the carbon dioxide acidifies the perfusate in order to maintain a physiological pH.

Each reservoir of perfusate can be individually selected and run through a roller pump, connected to a delivery system. The pump rate may be controlled directly, allowing delivery of a perfusate at a set rate ("constant flow perfusion"), or directed by a controlling computer system.

For these experiments, an AD Instruments Powerlab system was used, along with Chart5 for Windows (AD Instruments) running on a desktop computer connected to the Powerlab. This setup provided multichannel monitoring of flow and pressure. It also allowed for negative feedback control of the perfusion rate for those experiments in which pressure was controlled constantly.

The perfusate was delivered to a cannula; this cannula could be exchanged for others of differing sizes. It was important to match the size of cannula to the age of heart being perfused. For an adult heart, a 16G cannula was used; a 20G for 28 day old hearts, and for a 14- day old, a 24G cannula. As the flow rate was controlled by the pump, the change in resistance from these different cannula sizes did not impact upon the rate of delivery of perfusate.

For monitoring of the perfused heart, the system had two pressure transducers. One of these was connected to the column of fluid directly above the perfused heart. This was used to measure the perfusion pressure, and through feedback to the controlling Powerlab could be used to maintain the coronary perfusion pressure at a desired level through automatic alteration of the coronary flow rate.

The second of these transducers was connected to small bore flexible plastic tubing terminating in a fine blunted needle. Thin plastic film was folded over and around this needle to create a sealed balloon at the end; this when filled with ddH_2O was inserted into the LV of a perfused heart and would measure the LV pressure throughout the cardiac cycle.

Both of these pressure transducers were calibrated at the start of the series of experiments examining the effects of isoprenaline and adenosine on function and cardioprotection. They sensed a pressure on a membrane and transduced that into an electrical potential, which was amplified in the Powerlab and the magnitude of this amplified potential displayed. Each transducer had a calibration experiment performed to convert this potential into mmHg, the conventional unit of measurement for biological pressures. A vertical piece of flexible plastic tubing was used to apply pressure from a known height (in centimetres) of water to the pressure transducer. Values for each of 20, 40, 60, 80 and 100 mmHg were converted to cmH_2O and used to mark out points on the tubing. The tubing was filled to each of these marks with ddH_2O and the potential produced by each pressure transducer at each of these points measured. This was then repeated, and the mean voltage calculated. For each transducer, a standard curve was produced, allowing a conversion factor to be used to calibrate the sensor to produce an output in mmHg.

This experimental setup with pressure control proved problematic; with the immature hearts it was difficult to maintain a perfusion pressure as slight variation with the isolation and cannulation caused large changes to flow rate; and the act of inserting an intraventricular balloon itself caused multiple points of failure. Therefore, for the experimental sets carried out later using cAMP analogues, a constant flow technique without LV pressure monitoring was used.

2.3.3 Perfusion experiments

2.3.3.1 Constant flow perfusion

Once the heart was excised, the aorta was identified. This was incised to produce a clean edge, and then fine toothed forceps used to hold two opposing edges of the artery. Perfusate flow was started at a rate of 1ml/min, and then the aorta gently lifted onto the cannula and clamped in position such that the perfusate would flow in a retrograde direction towards the aortic root and from there down the coronary arteries. The heart was then secured in position using 2x 2-0 braided polyester sutures.

The flow rate was then gradually increased until reaching the target rate for each age group. For the 14-day old hearts, this was 4 ml/min, and for the adults, 10 ml/min, based upon previous work in our group.

2.3.3.2 Constant pressure perfusion

For experiments performed with constant pressure perfusion, once the heart was cannulated, the flow was started at a conservative rate of 3 ml/min in the immature group and 7 ml/ min in the adults. The pump was then switched to a pressure-linked negative feedback mode, and the set point slowly increased to the target perfusion pressures (80 mmHg for adults, 65 mmHg for 14- day olds). Once that target was reached, the Powerlab was switched to a pressure-hold mode, which maintained the current pressure sensed by the CPP sensor by alterations in flow rate in response to uncontrolled alterations in CVR.

If LV pressure was to be measured, an incision was then made into the left ventricle of the heart in order for the pressure transducing balloon to be inserted. Once inserted, it was inflated to a relative pressure of 0 -10 mmHg, measurement of the output from the transducer produced a pressure- time trace. The Chart5 software was programmed to derive several measurements from this trace;

-Maximum pressure reached

-Minimum pressure reached

-LVDP, which was calculated as the difference between these two measurements

-Heart rate, which was the number of pressure peaks detected per minute

At this point the hearts were allowed to equilibrate before being exposed to the experimental protocol, detailed in Chapter 4. For those experiments performed with constant flow, after cannulation the flow rate was set to 10 ml/min for adult hearts and 4 ml/min for 14-day olds. After equilibration, these hearts then followed their respective experimental protocols.

2.3.3.3 Protocols for studying function of Langendorff hearts with or without I/R

2.3.3.3.1 Intraventricular pressure monitoring

In experiments in which an LV pressure was monitored, a balloon was placed in the left ventricle. In order to do this, an arterial catheter (Vygon, Swindon, UK) was attached to a pressure sensitive transducer. This transducer was in turn monitored using the software Chart5 (AD Instruments). On the end of the catheter was fashioned a balloon from thin, flexible plastic of smaller volume than the ventricle to be studied and secured in place with a tied suture. The catheter and balloon was filled with dH_2O and the pressure adjusted so that the gauge pressure read close 0 mmHg on the transducer.

The pulmonary artery orifice was then identified on the surface of the left atrium once *ex vivo* heart perfusion was satisfactory, and opened slightly. The balloon was then gently placed into this opening, and passed in an apical direction to lie within the cavity of the left ventricle. If the pressure in the balloon interfered with the contraction of the ventricle, it was adjusted until this could no longer be seen to be the case.

Monitoring of the transducer then provided an LV pressure/ time trace throughout the experiment. Subsequent analysis using the software allowed extraction of data on systolic and diastolic LV pressures and developed pressure. Rate estimates could also be derived.

2.3.3.3.2 Coronary perfusion monitoring

In order to measure coronary perfusion pressure, a pressure sensitive transducer was attached to the perfusion apparatus directly above and in continuity with the perfusate. Prior to the start of cannulation and perfusion, it was set to 0 mmHg with reference to atmospheric pressure; the pressures it would measure were therefore in addition to atmospheric as is conventional in the measurement of physiological pressures.

The output from this transducer was measured digitally and converted to a pressure using the software Chart5 (AD Instruments). It was then used to drive negative feedback control of the perfusion pressure by alteration of the perfusion rate. This was set to 60 mmHg for experiments on P14 animals, and 80 mmHg for experiments on adult rats, which gave initial perfusion rates equivalent roughly to those used in constant flow setups.

2.3.3.4 Markers of necrotic injury

2.3.3.4.1 LDH Activity Assay

For all of the hearts used for Protocol 1 & Protocol 2, the effluent was analysed 24 hours later for LDH activity.

LDH catalyses the following reaction which is bidirectional depending on the concentration of substrates:

$$\text{Lactate} + \text{NAD} \leftrightarrow \text{Pyruvate} + \text{NADH}$$

Aliquots from the effluent were mixed with a reaction buffer as described by the manufacturer's instructions. This reaction buffer consisted of: Pyruvate 1.25 mM, NADH 0.11 mM & Triethanolamine 0.1 M and made up to 1L with ddH_2O and pH adjusted to 7.4 was used. When mixed with the LDH- containing effluent at 37° C, the optical density measured at 340 nM declines. For most of the reaction period, the pyruvate saturates the reaction kinetics of the LDH and so the change in absorbance is linear, with the rate of change of OD directly proportionate to the LDH activity.

The activity of the enzyme, measured in mU/ml is calculated using the following equation:

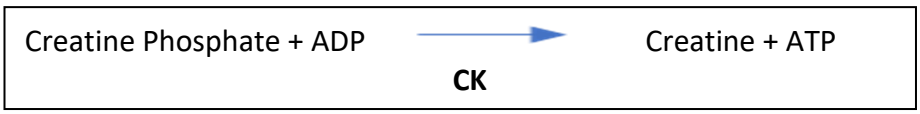
$$A = \frac{Observed\ gradient\ *\ 1000000}{6.22 * 80 * -1}$$

LDH is released when myocardial tissue is injured, and so the activity of LDH in coronary effluent may be used as a proxy measure of the degree of injury. Was this corrected for heart weight?

2.3.3.4.2 CK Activity Assay

For the experimental set involving perfusion of hearts with cAMP analogues, the coronary effluent was assayed for CK activity using a kit obtained from Randox (Crumlin, Co. Antrim, Northern Ireland).

Creatine Kinase is an enzyme released from cardiac tissue when damaged that catalyses the phosphorylation of ADP from ATP and creatine phosphate;



The resulting ATP is used to form glucose- 6- phosphate from glucose in a reaction catalysed by hexokinase;

Subsequently the G-6-P is oxidised to 6- phosphogluconate in a reaction reducing $NADP^+$ to NADPH catalysed by glucose-6-phosphate dehydrogenase (G6P-DH);

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G6P-DH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^{+}$$

The availability of substrate, hexokinase and G6P-DH is not limiting, whereas that of CK is, so the rate of formation of NADPH is directly proportional to the CK activity in the sample.

As this reaction proceeds, the absorbance at 340 nM changes. The rate of this change is determined spectrophotometrically; then this rate multiplied by a factor of 8095 (a conversion factor advised by Randox for use at 340 nM/37C) to obtain an activity measure in U/l.

2.3.3.5 Measurement of Area of Infarction

Figure 2-5. Example appearance of slices of heart stained with TTC following 30 minute global ischaemiaShown here, an Adult heart from the control protocol. Size reference 1cm x 1cm.

The infarct size generated by each protocol was determined by staining each heart with triphenyl tetrazolium chloride. A 1% TTC solution in PBS was used. 20ml was injected into each heart via the aortic cannula at the pre-terminal perfusate flow rate. The heart was then immersed in the TTC solution for 15 minutes, and then immersed in 10% formaldehyde for a further 15 minutes, before being removed and stored at -20° C. Infarcted areas appear white in colour whilst non-infarcted tissue is stained a deep red as shown in Figure 2-5.

The frozen hearts were then sliced with a #4 blade scalpel in a transverse direction into 5 slices for each heart, and the slices were scanned to produce images.

2.4 Isolated Cardiomyocytes

2.4.1 Cardiomyocyte Isolation

Isolation of cardiomyocytes required preparation in advance of several buffers for perfusion of the heart during and following digestion, of which Solution A (Table 2-3) formed the basis.

Perfusion with solution A is then followed sequentially by the solutions shown in Table 2-4. Perfusion is first switched to B and then to C; D performs digestion, then following recovery of the cardiomyocytes Ca2+ is sequentially reintroduced by sequential addition of solutions E, F, and G.

Constituent	Concentration
Solution B	750 µM CaCl2 & 16 mM Glucose
Solution C	90 µM EGTA & 16 mM Glucose
Solution D	10 mg/ml Type 2 Collagenase 1 mg/ml Protease
Solution E	150 µM CaCl2
Solution F	0.5 mM CaCl2
Solution G	1 mM CaCl2

*Table 2-4*The heart was then perfused with Solution A at a rate previously determined as appropriate for the animal's developmental stage & heart weight. Once the heart was judged to have become stable during perfusion with solution A- with regular contraction, and in any event not less than two minutes, perfusion was switched to solution B for two minutes then to solution C for four minutes.

Following this, the heart was then perfused with solution D. This is a collagenase & protease- containing solution, circulated continuously until an end point of digestion was reached. This was the point at which the heart became soft and very compliant to touch, indicating that an appropriate degree of enzymatic digestion had taken place. This took between 20 and 30 minutes to occur, and seemed to vary based upon the size of heart used for that particular experiment.

After digestion was judged to be complete, calcium was slowly reintroduced to the heart by perfusion with solution E (essentially A with the addition of 150 µM calcium), for 4 minutes. The heat was then placed in a dish, and gently pulled apart with

forceps. If the heart is well digested, this is easily accomplished. The fragments were then shaken in Solution E for 4 minutes at 37C, filtered, and then allowed to sediment for 8 minutes. The sediment contained cardiomyocytes in suspension.

The next stage in the isolation is to gradually increase the calcium concentration in the suspension whilst avoiding overload in the freshly isolated, and vulnerable, cardiomyocytes. The supernatant was pipetted away, and replaced with 5ml of solution F, which is solution A and 0.5 mM calcium. This is then replaced in a similar way with 5 ml of solution G- containing 1 mM calcium, 5 minutes later. As a final check, a small sample of the suspension was taken by Pasteur pipette onto a coverslip and examined under 20x magnification, to examine the morphology of the isolated cells. 5 µL of Trypan Blue dye was then added to the suspension; this dye will be taken up by cells in the suspension which do not have an intact cell membrane. Those which are intact, will not take up the dye and in this manner an overall viability rate of the cell suspension may be calculated.

2.4.2 Simulated Reperfusion in Superfused Cardiomyocytes

The cardiomyocyte cell suspension was divided into 5 ml aliquots into different conical flasks. These flasks of cardiomyocyte suspension were incubated in a water bath at 37^0C. Periodically, a droplet may be removed by Pasteur pipette and examined under a light microscope, having been stained with Trypan blue. This permits assessment of viability and morphology of the cardiomyocytes. Experimental protocols for these studies are described in Chapter 5.

2.4.3 Cardiomyocyte Superfusion

A droplet of the cardiomyocyte suspension was placed in a custom-designed microscope chamber through which a continuous constant flow of perfusate could be pumped. The cell suspension was allowed to settle and adhere onto the chamber surface for 10 minutes, before perfusion with buffer was started.

Initially, the cell suspension was perfused at 1.25 ml/min with a HEPES-based buffer warmed and maintained at 30°C; this was made up of the Solution A stock from the

isolation stage, as well as being made up to a concentration of 16 mM glucose and 2 mM calcium chloride in order to facilitate the assessment of contractility.

The cell suspension was then examined under light microscopy at 10x magnification (Nikon Eclipse TE2000-U). For subsequent assessment of contractile function, a cardiomyocyte had to be identified which was not only firmly adherent to the glass of the microscope chamber whilst the perfusate was flowing, but was also not arrhythmic (in this context, contracting without stimulation), and with a grossly normal rod- shaped morphology. The field was therefore searched to find a cell with these characteristics.

Once a suitable cardiomyocyte was identified under the microscope an electrical potential was then applied to the suspension in order to depolarise the cardiomyocyte being examined and stimulate contraction. This was performed using a GRASS SD9 Stimulator, delivering 100V stimulation at 0.2Hz, with a 1 ms delay between pulses which themselves lasted 4.5 ms.

2.4.4 Contractility Using an Edge Tracker

An Edge Tracker (Photon Technology International) was used to quantify the contractions in isolated cardiomyocytes. Alignment of this device's edge trackers on a display to the boundaries of the cardiomyocyte on the longest dimension allowed measurement of the degree of contraction relative to the initial size of the cell, providing a measurement of fractional shortening of the cell; typically this was on the order of around 10% at baseline. This method of measurement allowed a continuous trace of cell size vs time; and so contractile function during and after perfusion with alteration or addition to the buffer could be estimated.

2.4.5 Intracellular Ca^{2+} Measurements using Fluorescent Microscopy

The calcium transient through the contractile cycle in cardiomyocytes was also assessed, at baseline and following perfusion as before with cyanide and cAMP analogues. In order to accomplish this, the cardiomyocytes in suspension were first loaded with the fluorophore Fura-2-AM (2 µM) and pluronic acid (5 nM). Fura-2 binds to free calcium ions and in doing so its absorption spectrum changes- with a peak at

340 nm when bound to calcium, and 380 nm when unbound. Measuring the ratio absorbances at these wavelengths allows a relative quantification of calcium. This measurement may be carried out continuously, and has a relatively high temporal resolution, and so may be used to monitor free calcium levels in real time.

Once the Fura-2 had been added to the cardiomyocyte suspension, it was shaken in a water bath at 50 rpm at 37°C for 30 minutes. Subsequently, the cell suspension was separated in a centrifuge to remove the excess; at 100 RCF at 20°C for one minute. The supernatant was removed, and then the pellet resuspended in solution A. Care was taken throughout to minimize the exposure of the suspension to light, to minimize breakdown of the Fura-2.

2.4.6 Ca^{2+} Transient Measurements

The microscope chamber (as described in the section on cardiomyocyte contractility measurements) was continually perfused with Solution A. A drop of the Fura-2 cell suspension was added, and perfusion stopped for ten minutes. This period of no flow allowed time for the cells to adhere to the glass of the microscope chamber; with the aim of enhancing stability during the period of study.

After this period, the field was examined for a cardiomyocyte which retained a rod-shaped morphology, was fixed onto the slide- did not move with perfusate flow, and which responded to depolarisation with a stimulating field (0.2 Hz at 100 V, pulse with of 4.5 ms as previously) and did not show any visible contraction without stimulation i.e. was not dysrhythmic. Perfusion flow was then recommenced at 1.25 ml/min. The microscope and equipment were then enclosed in a curtain to prevent bleaching from outside light sources.

The first step in measuring transients by fluorescence was then a quantification of background light; a measurement of this intensity was taken over 30 minutes to arrive at an average background intensity. The analysis software then subtracted this background from subsequent measurements of the cardiomyocyte, in the hope that this would allow a more accurate measurement of the fluorescence from calcium alone.

Simultaneous measurements of emission intensities were then taken over time whilst the cardiomyocyte was being stimulated and illuminated at 510nm using a PTI photomultiplier (Model 814). A reading at 340 nm represented bound calcium, whilst another at 380 nm for free calcium. A ratio of these two values was then plotted, which modelled the relative intracellular calcium concentration in real time.

2.4.7 Experimental Protocols in Isolated Cardiomyocytes

2.4.7.1 Protocols for measuring effect of cAMP analogue on cardiomyocyte contractility

Following identification of a stably contracting cardiomyocyte, a measurement of its baseline contractility was taken. Perfusion then switched to a buffer made up of Solution A, glucose, and calcium chloride as before but with the addition of 2.5 mM sodium cyanide. The Cyanide ion is an irreversible cytochrome oxidase inhibitor, and so causes a reduction or cessation in oxidative phosphorylation flux in a dose dependent manner; it is thus a cause of histotoxic hypoxia and may be used as a model of acute hypoxia. Perfusion with the cyanide- containing buffer was continued for 30 minutes, and the time to development of arrhythmia, the time to cessation of contractions and the time to cell death (sudden & irreversible change in cardiomyocyte morphology from elongated rod-shaped to spherical) was measured as the end- points.

In order to determine the contributions of signalling flux through PKA & EPAC to cardiomyocyte contractile function, these experiments were repeated with the prior perfusion of engineered cAMP analogues. The cardiomyocytes were perfused with the buffer containing either a non-selective EPAC & PKA agonist (8-Br-cAMP-AM), or either a selective agonist of PKA (6-Bnz-cAMP-AM) or EPAC (8- (4- CPT)-cAMP-AM) (all from BioLog Life Science Institute, Germany). These cAMP analogues were the 'AM' version- indicating that they are conjugated to an acetoxymethyl group which increases the permeability of the molecule across the cardiomyocyte cell membrane. The concentrations of these agents were chosen so as to be consistent with prior

work undertaken for this thesis and in the group, detailed in Khaliulin *et al*., 2017 (Khaliulin, Bond et al. 2017) at 5 µM for 8-Br, and 10 µM for CPT and 6-Bnz.

2.4.7.2 Protocol for measuring effect of cAMP analogues on Ca^{2+} transients

After a period where a seemingly stable cardiomyocyte was allowed to contract with a drug-free buffer and baseline calcium transient measurements obtained, the perfusate was switched. The cardiomyocyte was perfused with a cAMP-analogue (one of the three agents detailed in Table 2-8) containing solution for 5 minutes, followed by a 30 minute period of perfusion with a buffer containing Solution A, 2.5 mM sodium cyanide, and 2.5 mM Ca^{2+}. Calcium was measured continuously through this 30 minute period or until death of the cardiomyocyte or movement of the cell from the field of view under the microscope occurred.

2.4.7.3 Protocols for measuring cardioprotective efficacy of incubated cAMP analogues & CsA on isolated myocytes in suspension

Each of 5 ml conical flasks containing cardiomyocytes was used as an independent experimental arm; the first of each was used as a control. Flasks 2 and 3 both were made up to 0.5 mM H_2O_2 and 3 mM $CaCl_2$, whilst Flask 3 had 2 µM cyclosporin A (CsA) added to the suspension for 10 minutes prior to the addition of hydrogen peroxide and calcium or one of the cAMP analogues. The control arm served as a baseline monitor of changes in cell viability after cardiomyocyte isolation without further intervention; whilst the addition of hydrogen peroxide and high calcium aimed at simulating the conditions present during reperfusion following ischaemia. Each of these experimental conditions were then incubated and agitated in a water bath at 37C for 120 minutes.

Each of these flasks were sampled at 30 minute time points following the start of the experiment- from a sample taken at the outset as a baseline, to a final sample at 120 minutes. When sampled, a drop was placed by Pasteur pipette onto a microscope coverslip, and 5 µl of Trypan blue added. This enabled an estimation of the proportion of viable cardiomyocytes at each sampling interval, as well as their morphology (rounded vs. rod shaped).

2.5 Isolated Cardiac Mitochondria

2.5.1 Cardiac Mitochondrial Isolation

Following Langendorff perfusion, the heart was removed from the aortic cannula and washed in Buffer B. The atria and accessory tissues were removed, and the remaining tissue cut rapidly into small pieces. The tissue was then immersed in 3 ml of Buffer A and transferred to the cold room for homogenisation at 4^0C.

The heart was then homogenised using a Kinematica Polytron device, at a speed of 7000 rpm for 4 bursts of 5 seconds. The probe was then washed with 5 ml buffer B, which was added to the homogenate. The homogenate was then made up to a total volume of 30 ml with buffer B. This suspension was separated using a centrifuge at 2000 g for 90s; the supernatant was retained.

This supernatant then underwent further centrifugation; at 10,000 g for 5 minutes (Beckman Optima L-90k ultracentrifuge with a Type 45Ti rotor- this was equivalent to 11,300 rpm). The supernatant from this separation was discarded, and the pellet resuspended in 1 ml of Buffer A final wash. This suspension was then made up to 30 ml. A further centrifugation step at the same speed and duration was then used, and the pellet then suspended in 200 µl of Buffer AFW; this was considered then to be a suspension of mitochondria.

2.5.2 Determination of Mitochondrial Protein Concentration

As the subsequent mitochondrial assay described below should be undertaken using a known and standardised concentration of mitochondria, a Bradford assay was used to estimate the concentration in the mitochondrial suspension.

5 mg/ml Bovine Serum Albumin (BSA) was diluted in Buffer AFW to a series of standards; 0.5 mg/ml, 1 mg/ml, 2.5 mg/ml, and 5 mg/ml. These standards of known concentration would form standard curve of absorbances. A sample of the mitochondrial suspension was diluted 10x in Buffer AFW in order to place the measured absorbance within the range of the standard curve.

3 ml of Bradford reagent was added to 10 µl of each standard and the mitochondrial solutions, mixed, and the reaction allowed to proceed for 15 minutes at room temperature. The absorbance was then measured using a spectrophotometer at 595 nm.

Using the absorbances of the concentrations from the known standards, a standard curve was plotted. Then, by linear regression, the estimated concentration of the mitochondrial suspension estimated by the measured absorbance.

2.5.3 Determination of MPTP Opening Using a Spectrophotometer

Mitochondrial suspension in swelling buffer (0.2mg/ml) was prepared by adding 1.4 mg of mitochondria to 7 ml swelling buffer prewarmed to 30^0C. This suspension was then divided into 2 x 3.5 ml aliquots in two large cuvettes. These cuvettes were then placed into the experimental and control chambers of a Thermo Scientific Evolution 201 spectrophotometer, which allows simultaneous real time comparative analysis of absorbance between the two chambers.

Initially, a baseline recording of absorbance at 520 nm was taken, with data being recorded at 2 Hz. This recording was made for 120 seconds and allows the baseline rate of mitochondrial swelling to be measured without intervention.

Subsequently, $CaCl_2$ was added to the experimental cuvette to give a calcium concentration in the mitochondrial suspension of 3 mM for experiments with non-ischaemically derived mitochondria, or 1 mM for those isolated from experiments involving I/R. The use of 1 mM Ca^{2+} is conventional in this method of assessing pore sensitivity (Abellán, Miró-Casas et al. 2006, Khaliulin, Parker et al. 2010); it allows detection of an effect within the sensitivity range of the spectrophotometer. Conversely, the use of 3 mM Ca^{2+} in uninjured hearts is less well described. Initial experiments using the lower Ca^{2+} dose showed a small effect, and so in the optimisation of these experiments the use of 3 mM Ca^{2+} allowed heightening of the effect size when the sensitivity is low.

The recording was then restarted and continued for at least 300 seconds or until any change in absorbance had ceased in order to measure the change in absorbance characteristics that accompanies swelling of the mitochondria as a consequence of MPTP opening triggered by the addition of Ca^{2+}.

2.5.4 Protocols for measuring the effect of cAMP analogues on mitochondrial swelling

Hearts from adult, male Wistar rats (Charles River Laboratories) were perfused in the Langendorff mode using Krebs- Henseleit buffer (Table 2-2) with 95% O_2/5% CO_2 bubbled through, maintained at a temperature of 37.5 ^{0}C. Once each heart had been established in the perfusion setup, an experimental protocol was started. Several protocols were used;

1) Perfusion of the heart for 30 minutes before termination of perfusion; a control experiment without ischaemia/ reperfusion injury
2) Perfusion of the heart for 30 minutes followed by a 30 minute period of global ischaemia, then a 5 minute reperfusion period before termination of the experiment; a control experiment with ischaemia/ reperfusion injury
3) Perfusion of the heart for 30 minutes followed by perfusion with a cAMP analogue (each from *Table 2-8*) for 5 minutes, then a washout period for 5 minutes, followed by a 30 minute period of global ischaemia, then perfusate flow restarted for 5 minutes as a period of reperfusion before the experiment was terminated.

Each of these experimental protocols was tried in order to show differences in the opening characteristics of the MPTP, both under control or physiological conditions or with perfusion with a cAMP analogue. In each case, after the perfusion step of the experiment, the rest of the investigation was performed identically.

2.6 Statistical Analysis

2.6.1 Proteomics Statistical Analysis

Each proteomic run of experiments produced an abundance for each peptide for each sample. Results with a low or medium level of confidence in interpolation of parent protein from the measured peptide were rejected.

For comparisons between P14 proteins and adult, significance levels were estimated by t- test with Bonferroni *post hoc* correction. One- way ANOVA was performed where multiple groups were compared.

The data were then presented in the form of a logarithmic plot comparing the mean fold change against the significance level of that change, such that significant changes of notable difference could be identified; this should produce identification of both statistical and biologically significant differences.

2.6.2 Data from Langendorff perfused hearts

2.6.2.1 Processing of Langendorff perfused heart contractility data

Where LV pressure/ time data was collected, it was used to calculate developed pressure as well as systolic and diastolic pressures. The pressure changes with drug treatment were compared with baseline pressures immediately preceding. Statistical analysis of these differences were performed by Student's t-test and reported as significant if $p < 0.05$.

Rate-pressure product was also determined by analysis of the same trace. Similarly comparison was made immediately before addition of a drug and at maximal effect; comparisons were made by Student's t-test and reported as significant if $p < 0.05$.

2.6.2.2 Processing of Enzymatic release from perfused heart data

An enzyme activity measure (of CK or LDH) was estimated for each 5 minute reperfusion fraction, and normalised to the perfusate flow rate at the time of sampling and to the mass of tissue perfused. Each fraction was compared by Student's t-test. The area under the activity- time curve was also estimated geometrically, to allow for comparison across the reperfusion period.

2.6.2.3 Processing of area of infarction data

The proportion of the heart that was infarcted was determined planimetrically using the software ImageJ (Abramoff 2004) (National Institute of Health, Bethesda, United States). The whole heart is at risk of infarct in global ischaemia, and so the infarcted proportion is the size ratio of the infarcted area vs. the area of the whole heart; therefore the magnitude of the infarct could be expressed in percentage form. Comparisons between experimental groups were then made by Student's t-test between intervention groups and the same age group control.

2.6.3 Data from Isolated Cardiomyocytes

2.6.3.1 Processing of Isolated Cardiomyocyte Contractility Data

For each contracting cardiomyocyte trace, time points at 5 minute intervals from the start of the experiment until 35 minutes were examined. Cardiomyocyte size values from a window of 15 seconds either side of the instantaneous time point were assessed; this would reliably capture at least 3 whole contractile cycles at the stimulation rate of 2 Hz. For those cycles, maximal and minimal output values were selected and converted to actual sizes through an initial calibration and a mean value for the maxima and minima taken across the cycles observed. This was then expressed as fractional shortening, and then normalised to the initial value for that cardiomyocyte. Comparisons were then made by two- tailed, unpaired t-test against the data at 5 minutes and 35 minutes for the cyanide- alone control to determine the effects of the cAMP analogues on the unpoisoned cardiomyocyte (at 5 minutes) for the baseline pharmacological effect, and after 30 minutes of cyanide perfusion (35 minutes after the start of the experiment) representing protective efficacy in this model of hypoxia.

2.6.3.2 Statistical processing of calcium transient data from superfused myocytes

Calcium transients (Fura ratio) were computed from the diastolic and systolic Ca^{2+} in electrically stimulated cardiomyocytes throughout the superfusion experiments. Data were sampled at 5-minute intervals throughout each experiment- from 0 to 25 minutes, and exported from the imaging software for analysis. At these time points,

the bound/free calcium activity was calculated and then expressed as a ratio of the baseline at the t=0 timepoint.

For each cell studied, 3 contractile/transient cycles either side of the selected time points were sampled, and the mean calculated as well as the standard error of the mean as a measure of spread. Comparisons by t-test were made of the data at 5 minutes (for the physiological effects of the cAMP analogues) and at 25 minutes (for preservation of calcium transients despite histotoxic hypoxia from the NaCN perfusion).

2.6.3.3 Statistical processing of data from cardiomyocytes in suspension

Each experimental condition was measured at 30-minute intervals. 5 random fields were selected from a droplet examined under 20x light microscopy and photographed. Each of these fields were subsequently assessed; the number of dead/ hypercontracted/ normal cells counted and a mean value/ SEM calculated for each group at each time point. Between group comparisons of viability and morphology were calculated at each time point by two- way repeated measures ANOVA with a Bonferroni post-hoc modification.

2.6.4 Statistical processing of data from isolated mitochondria

Each experiment resulted in a trace of absorbance at 520 nm vs. time, with a baseline trace and a trace after the addition of supplemental Ca^{2+}. For each experiment, the Ca^{2+} induced swelling trace was corrected for the background rate of drift. Then, the maximal rate of change of absorbance (dA/dt, over a 3 second window of recording) and the absolute difference between baseline and experimental arms was measured to estimate the opening sensitivity of the MPTP in each group. A two- way ANOVA was then performed with Tukey's post-hoc test of significance to assess the differences between the age groups and treatments studied.

3 Molecular Developmental Changes Associated with I/R injury and cAMP/PKA/Epac signalling

3.1 Introduction

The information provided in the Introduction suggest that signalling downstream from cAMP by alterations in activity and targeting of intermediates may, at least in part, explain the finding that the immature heart differs in its sensitivity to ischaemia/ reperfusion injury compared to the adult. Therefore, elucidating and identifying the proteins involved in cAMP signalling pathways would help to understand the difference in vulnerability.

The field of proteomics, in concert with sister disciplines in genomics, metabolomics, transcriptomics amongst others has emerged over the last two decades due to parallel advances in technology allowing for the identification and identification of complex organic molecules rapidly, with precision, and from a relatively small sample. These techniques allow rapid and precise assessment of how the scope of the whole proteome changes across a range of experimental conditions; rather than being limited to studying one or a small number of proteins.

3.2 Aims

There are three aims for the experiments described in this chapter.

1. To examine the comparative cardiac proteomes of rats at various stages of maturation and identify thematic changes over that period of development.
2. To look in detail at the cAMP signalling axis where it appears in the proteome and use that to examine the differences that are supposed based up on the results evident in the previous Chapter, on whole heart perfusion.

3.3 Methods

The techniques for extraction, digestion, mass spectrometry and peptide identification are discussed in the Methods chapter of this thesis, but are reiterated here in brief.

Rats of 7 (n=4), 14(n=9), and 28(n=9) days postnatal as well as adults(n=9) were killed by stunning by occipital concussion and then by cervical dislocation. Their hearts were rapidly extracted and agitated in chilled cardioplegia buffer. A small piece of tissue from the apex of each heart was cut off and snap frozen in liquid nitrogen, then stored at -80 °C for later use.

The samples were then homogenised, and the protein content from each sample of tissue was then extracted. This was performed by rapid oscillation in RIPA buffer, followed by centrifugation at 10,000 x g. The supernatant in which the extracted protein content was in solution was then recovered. The concentration of the protein in the supernatant was quantified by Bradford assay, and then normalised at 2mg/ml.

The samples were then analysed at the University of Bristol Proteomics facility with the assistance of Dr. Kate Heesom. They were assessed through a TMT-MS/MS protocol for identification of unmodified peptides. This involved multiple sample runs through the mass spectrometer. Each of these experiments was limited to 10 samples at a time. The number of the original extracts that could therefore be used in a limited number of runs, and provide intra-experimental comparison and cross run validity was therefore limited. As each run required a pooled sample of all extracts in order to provide cross-run normalisation, there were 9 experimental samples per run. There were three runs performed, with age groups scattered across each run randomly in order to prevent an experimental bias. This limited the total number of samples to 27 (3 runs of 30 – 3 pooled samples altogether). The total number of extracted hearts per age group ultimately studied is shown in Table 3-1. The samples excluded were chosen randomly by the proteomics facility.

Age Group	*Number of hearts used*
P7	4
P14	8
P28	8
Adult	7

Table 3-1 Numbers of hearts used to produce protein extracts for each age group. Each heart produced one sample, which was independently run through the TMT-MS/MS process.

A bioinformatic approach then assigned those peptides to their likely protein of origin, with an estimated degree of confidence. The results were returned as an abundance relative to that in a pooled sample. The first stage of analysis was performed in Proteome Discoverer 2.1 (Thermo Fisher Scientific, Massachusetts, US), which returned a list of proteins as well as their abundance per sample and in the pooled sample. The functional category of these proteins was assessed over the whole proteome by age group by importing the list of proteins from each age group into the categorisation tools at PantherDB.org. This provided a breakdown by function of the proteins identified in each age group.

The 14- day old results were then selected and compared with the results for the adult age group. Statistical comparison between these age groups was performed by Student's t-test and Bonferroni correction; a fold- change was also calculated dividing the mean abundance in the P14 group by the mean abundance in the adult group- so a fold change greater than 1 represented an increase in the P14 vs. the adult, and a fold change less than 1 the reverse. These two values, of p-values and fold change were then plotted logarithmically against one another for each protein to produce a 'volcano plot'; a graphical representation of the entire proteome- Figure 3-3, for example.

Subsequent analysis also performed in Ingenuity Pathway Analysis (Qiagen Bioinformatics, Hilden, Germany). This software allowed assessment of whole interacting networks of proteins rather than individuals; and produced scores for these networks reflecting their overall change in the age groups compared.

3.4 Baseline proteomics

3.4.1 Characterisation of Output

Each of the three runs involved different samples, and a different proportion of each age group was represented; consequently, a differing subset of proteins was identified from each run.

The results of these experiments were combined and demonstrated 6976 unique proteins across all three experimental runs, although not all of these proteins were identified in all samples or in all age groups; only 46% were common to all groups and experimental runs. Whilst in large part the identities of proteins identified was the same between groups, there was still considerable heterogeneity. Figure 3-1 shows graphically the age groups with uniquely identified and overlapping proteins and Table 3-2 a comparison of number of proteins in common between two age groups. Once those results with missing values were excluded, if all samples were analysed together there were 5038 unique proteins common to all groups representing 73.9% of the total proteins identified in at least one sample of at least one age group.

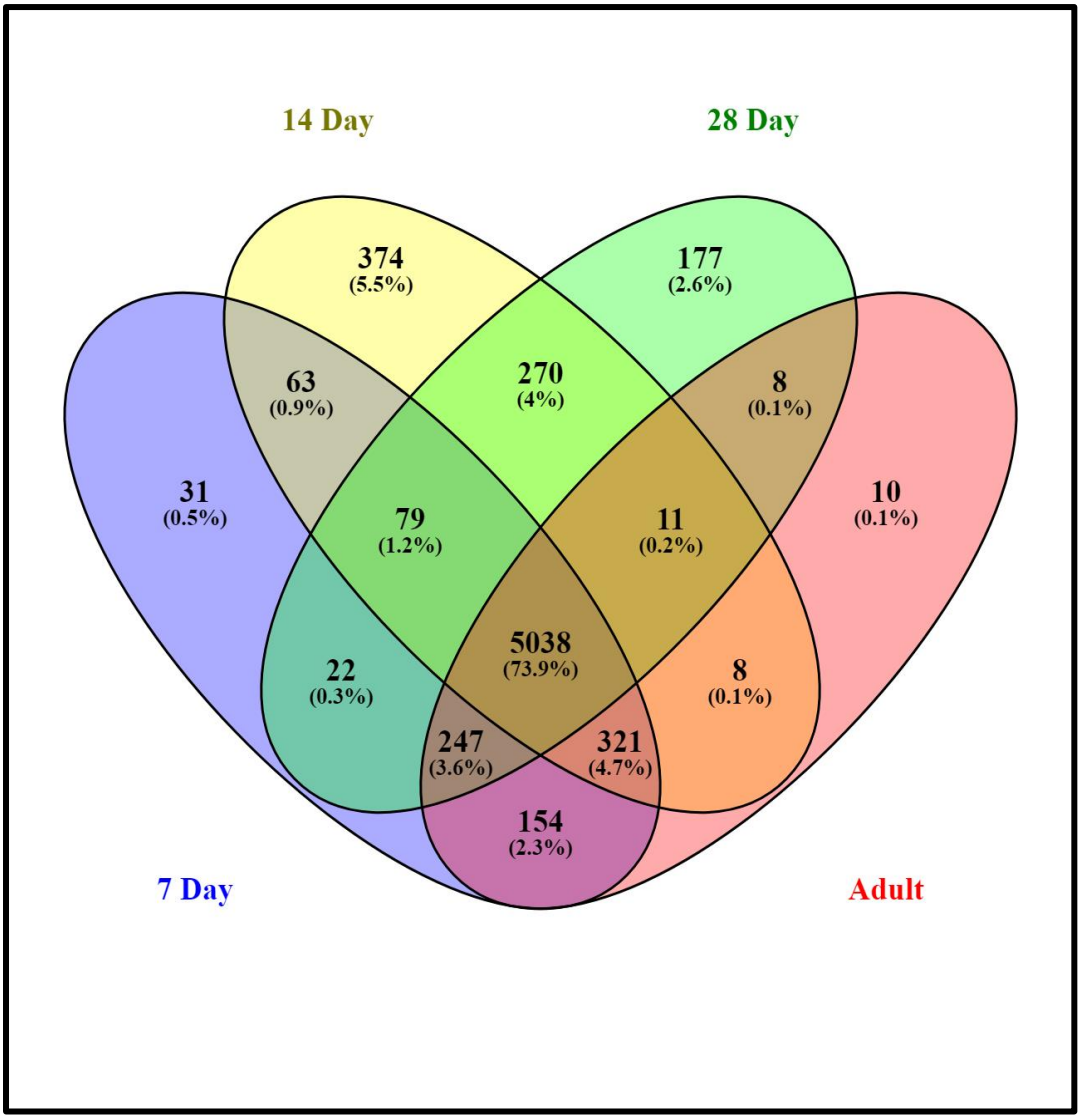


Figure 3-1. Venn diagram showing numbers of proteins identified from each experimental group with high confidence. Adult group n= 7; P28 group n=8; P14 group n=8; P7 group n=4.Overlapping areas show proteins extracted in common between each different age group. No details for expression levels nor significance testing were included in this analysis. (Oliveros 2007-2015)

In order to further characterise the proteome and gain an overview of how these broke down functionally, the accession numbers of the identified proteins were queried against a database of GO Molecular Function terms (Ashburner, Ball et al. 2000, Consortium 2015) using the tools available at PantherDB.org (Mi, Poudel et al. 2016), cross-referencing to the UNIPROT database (2015). The results are shown in Figure 3-2 and Table 3-3.

Comparison	*Proteins identified in all samples of group*	*Proportion of all uniquely identified proteins*
7 vs adult	4578	65.6%
14 vs adult	4040	57.9%
28 vs adult	4081	58.5%

Table 3-2. Numbers of proteins identified per age group vs. total proteins identified. Not every sample from each age group could be shown to have all the proteins that were identified in any sample.

GO Molecular Function Term	*Number of Proteins*			
	7 Day	14 Day	28 Day	Adult
Transporter Activity (GO:0005215)	180	203	188	177
Translation Regulator Activity (GO:0045182)	49	53	50	49
Catalytic Activity (GO:0003824)	1320	1352	1308	1291
Receptor Activity (GO:0004872)	56	62	59	54
Signal Transducer Activity (GO:0004871)	56	66	60	54
Antioxidant Activity (GO:0016209)	17	18	19	17
Structural Molecule Activity (GO:0005198)	216	212	214	215
Binding (GO:0005488)	1182	1235	1168	1154

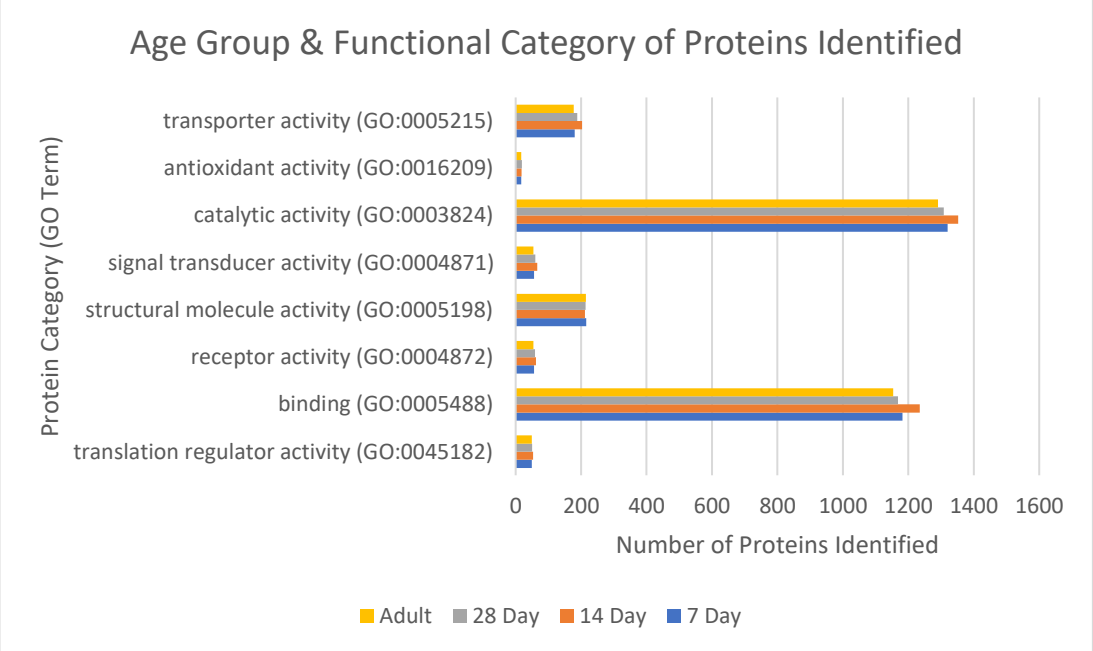


Figure 3-2 & Table 3-3. Proportion of proteins identified from each age developmental age group classified into functional groups according to the Gene Ontology Consortium's Molecular Function Terms. Adult group n= 7; P28 group n=8; P14 group n=8; P7 group n=4.

Broadly, these categories were very similar in frequency across the age groups- so there did not seem to be an age-related bias in types of proteins detected by this method. The proteins identified are not identical to the complete proteome, and so the possibility for significant systematic bias exists; it is therefore important that the age groups appear similar in this regard. This analysis thus far has not considered abundance of those proteins identified, which is where significant variation may appear.

3.5 Comparison of 14- day and Adult Proteomes

In rats, 14- day old hearts are thought to be the group most resistant to injury compared to those of adults (Introduction 1.6.3). These groups were therefore selected to be directly compared against one another in order to attempt to find potential explanations for this observation, in order to make the analysis more tractable.

A 2- tailed heteroscedastic Student's T-test with a Bonferroni post-hoc correction for multiple testing was performed across all the proteins identified in common from the 8 14- day hearts and 7 adult hearts comparing the significance of their relative abundances. The relative/ proportionate change was also estimated for each protein. It was decided based upon convention in the proteomic literature to regard a fold-change of >1.2 fold in either direction as potentially of interest.

	Fold change >1.2 (more abundant in 14 –day old)	Fold change <0.8 (more abundant in adult)	p <0.05
n. proteins	3740 (75.4%)	402 (8.1%)	3296 (66.5%)

Table 3-4. Number of proteins left in results after fold change and significance filter applied to proteins identified in all 14- day old and adult samples. Parentheses –proportion of all proteins.

All proteins which were found to meet the statistical significance threshold also met the fold- change threshold, so this analysis did not significantly reduce the number of candidate proteins to study. Subsets of interest of this proteome were therefore identified in order to facilitate analysis.

The GO terms described earlier provide a straightforward and relatively unbiased means by which to filter the data set, albeit dependent upon the validity of the annotation in the databases. Given that the 14- day old hearts display the greatest degree of resistance to injury, based upon previous experimental work, it was decided to focus the analysis on those sets and use the well-described adult phenotype as a comparator. The whole 14- day and adult proteome was annotated with GO terms for Molecular Function as well as subcellular localisation which permitted selection of subsets of interest. The proteome was searched for the terms

"channel", "calcium", "signal*" "redox" and the location "mitochond*" as these parameters were felt to delimit the search to those factors known to be of importance in I/R injury.

3.5.1 Calcium- related proteins

This group consisted of 296 proteins. From those, 184 were over the p-value threshold on Student's T-test of 0.05. All aside from 30 were greater than the 1.2 fold difference from the counterpart set. Selected proteins of particular interest, either from prior observation or standing out as an unexpected or particularly marked change, are highlighted in Figure 3-3 and Table 3-5.

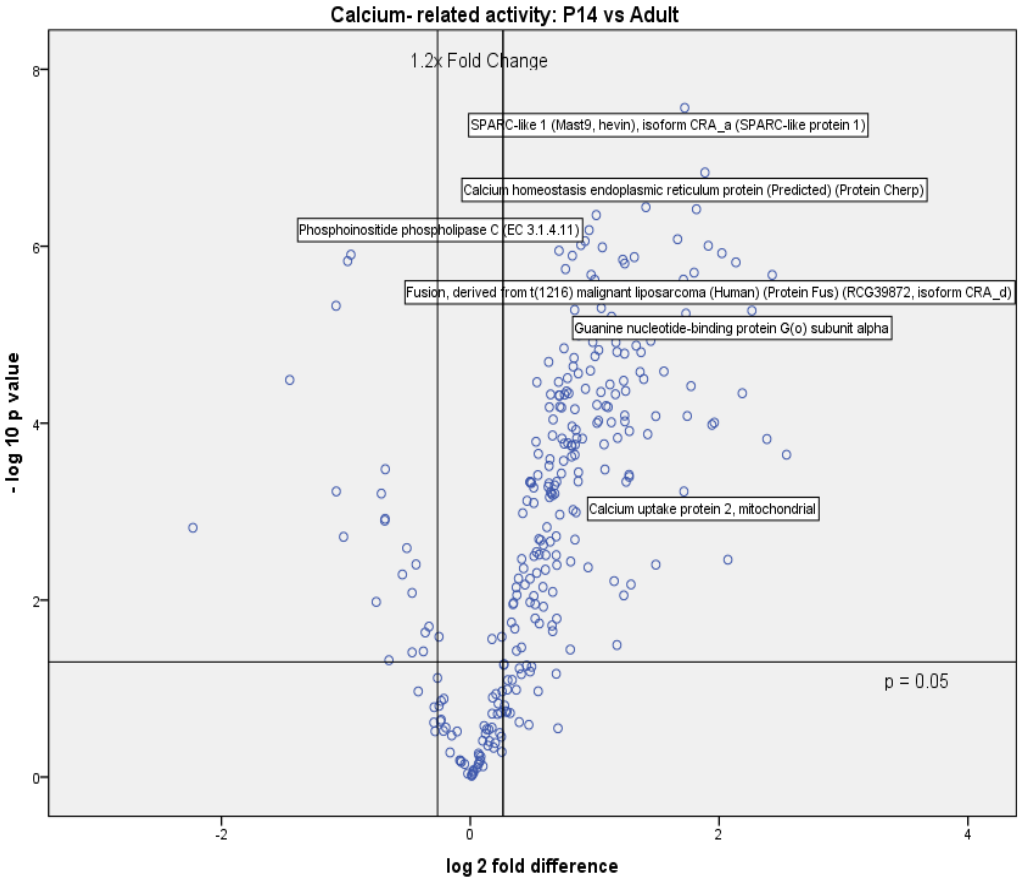


Figure 3-3. Logarithmic plot of fold change of Calcium- related dataset identified in 14 day- old hearts compared to adult samples vs. p- value derived from Student's T-test. Bonferroni post-hoc correction for multiple testing used. Adult group n= 7; P14 group n=8.Reference lines denote a fold change < 0.8 or >1.2 fold on the x- axis, or a p- value < 0.05 on the y- axis. Positive values of fold change represent an increase in the 14- day heart.

Protein Name	Accession	Fold Change (14-day/Adult)	p- value
Calcitonin gene-related peptide type 1 receptor	Q63118	3.766	0.000
Calcium homeostasis endoplasmic reticulum protein	D3ZAX5	3.697	0.000
Calcium uptake protein 2, mitochondrial	F1LMJ8	3.287	0.001
Sodium/calcium exchanger 1	Q01728	2.733	0.000
Calcium/calmodulin-dependent protein kinase II inhibitor 1	Q9JI15	2.265	0.000
Sodium/hydrogen exchanger 1	P26431	1.773	0.001
Ryanodine receptor 2	B0LPN4	1.619	0.016
Cardiac phospholamban	P61016	1.586	0.000
Cardiac-type ryanodine receptor	D7UNU1	1.583	0.008
Calcium/calmodulin-dependent protein kinase type II	P15791	1.543	0.001
Caveolin-1	P41350	1.461	0.000
Calcium channel, voltage-dependent	A0A0G2JSZ0	1.442	0.000
Calcium-transporting ATPase	D3ZH00	1.372	0.001
Sodium/potassium-transporting ATPase	P07340	1.308	0.006
Cardiac-type ryanodine receptor	D7UNT4	1.296	0.009
Calmodulin	P62161	1.273	0.011
Cardiac troponin C	Q4PP99	0.770	0.038
Troponin I, cardiac muscle	P23693	0.609	0.001
Sodium/potassium-transporting ATPase	P06686	0.474	0.001

Table 3-5. Selected proteins of interest from the calcium- related dataset. Fold change= ratio of relative abundance of mean P14 result to mean Adult result. P- value is the result of a two- tailed Student's T- test comparing the two groups with the Bonferroni correction for multiple testing. Sorted by order of decreasing fold change. Positive values of fold change represent an increase in the 14- day heart. Adult group n= 7; P14 group n=8.

3.5.2 Signalling Proteins

Here, 822 proteins were identified. Of these, 654 reached the significance threshold of p >0.05 on a Student's T-test; the majority of these 654- 611- were more abundant in the 14- day old heart, with only 43 more abundant in the adult heart. In all of these cases their fold change was >1.2. Selected proteins, either based upon prior observation or those that were unexpectedly up/downregulated, are highlighted in Figure 3-4 and Table 3-6.

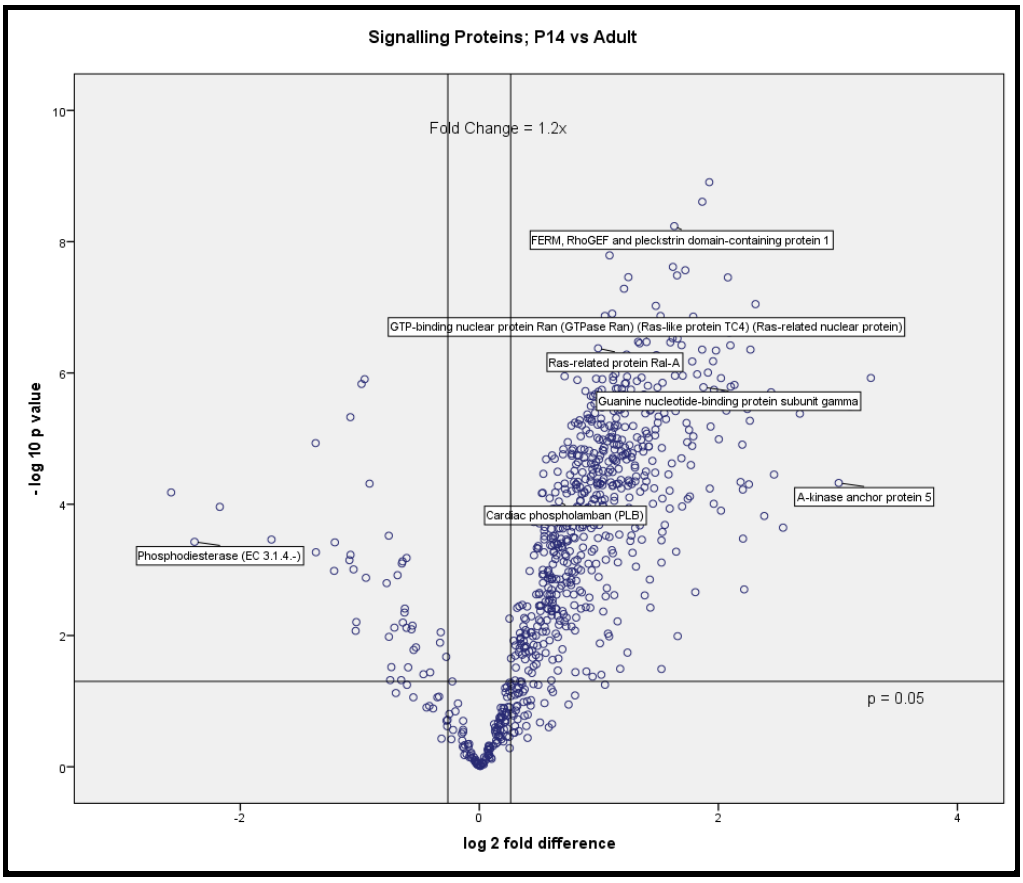


Figure 3-4. Logarithmic plot of fold change of signalling subset of proteins identified in extracts from 14-day old hearts compared to adult samples vs. p- value derived from T-test. Reference lines denote a fold change < 0.8 or >1.2 fold on the x- axis, or a p- value < 0.05 on the y- axis. Positive values of fold change represent an increase in the 14- day heart. Adult group n= 7; P14 group n=8.

Protein Name	Accession	14day/Adult fold Change	p- value
A-kinase anchor protein 5	F1LPP6	8.041	0.000
A kinase (PRKA) anchor protein 1	D4A9M6	2.670	0.000
Rho-related GTP-binding protein RhoB	P62747	2.606	0.000
G kinase-anchoring protein 1	Q5XIG5	2.581	0.000
Adenylate cyclase 6	F1LSD1	2.562	0.000
Phosphoinositide phospholipase C	G3V845	2.159	0.000
Protein Rap2a	A0A0G2JTW1	2.052	0.000
cAMP-dependent protein kinase	P68182	2.036	0.000
Phosphatidylinositol 3-kinase	O88763	1.997	0.001
A-kinase anchor protein 6	Q9WVC7	1.952	0.000
Phosphoinositide phospholipase C	A0A096MJW5	1.939	0.000
Protein kinase C epsilon	P09216	1.802	0.001
Caveolin-3	P51638	1.789	0.000
Protein Akap9	F1LPB4	1.752	0.000
Rho guanine nucleotide exchange factor 2	Q5FVC2	1.708	0.007
Caveolin	A0A0A0MXU8	1.506	0.001
Caveolin-1	P41350	1.461	0.000
A kinase (PRKA) anchor protein	F1M3G7	1.283	0.020

Table 3-6. Selected signalling- related proteins of interest. Fold change= ratio of relative abundance of mean P14 result to mean Adult result. P- value is the result of a two- tailed Student's T- test comparing the two groups with the Bonferroni correction for multiple testing. Sorted by order of decreasing fold change. Positive values of fold change represent an increase in the 14- day heart. Adult group n= 7; P14 group n=8.

3.5.3 Mitochondrial Proteins

816 proteins were identified with the mitochondrial cellular localisation GO term. This is not synonymous with the proteins having been extracted from the mitochondria, just that they are known to localise to the organelle but not (necessarily) exclusively. 557 of these met the significance threshold, of which 465 were 1.2 fold or greater more abundant in the 14- day old group. Only 82 of these were more abundant (>1.2 fold) in the adult group.

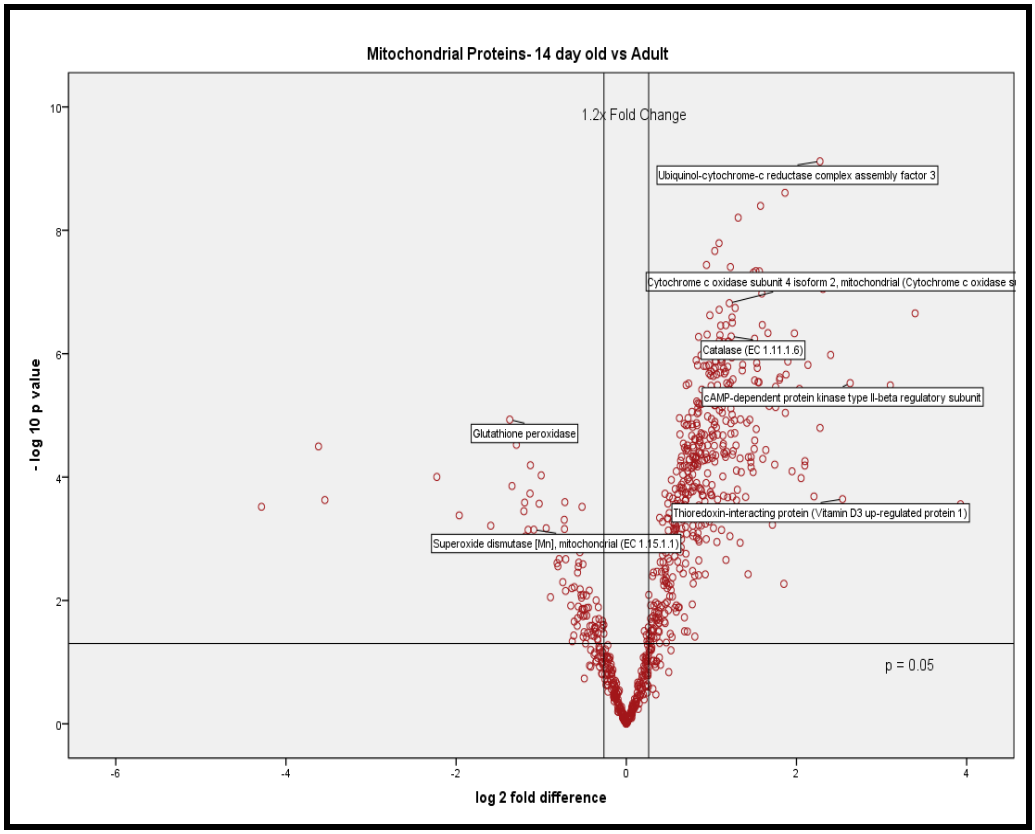


Figure 3-5. Logarithmic plot of fold change of "Mitochon" subset of proteins identified in 14-day old hearts compared to adult samples vs. p- value derived from T-test. P- value is the result of a two- tailed Student's T- test comparing the two groups with the Bonferroni correction for multiple testing. Positive values of fold change represent an increase in the 14- day heart. Adult group n= 7; P14 group n=8. Reference lines denote a fold change < 0.8 or >1.2 fold on the x- axis, or a p- value < 0.05 on the y- axis.*

Selected proteins of interest, either of those that are previously known to be significant, or those that were significantly down/ upregulated unexpectedly, are shown in Table 3-7 and Figure 3-5.

Protein Name	Accession	14 v Adult fold change	P- Value
Ubiquinol-cytochrome-c reductase complex assembly factor 3	P0CD94	4.843	0.000
Peroxiredoxin-4	Q9Z0V5	2.393	0.000
Catalase	P04762	2.349	0.000
Cytochrome c oxidase	P10818	1.791	0.000
Glutaredoxin-1	Q9ESH6	1.757	0.000
NADPH:adrenodoxin oxidoreductase, mitochondrial	P56522	1.496	0.001
NADH dehydrogenase	G3V644	1.395	0.006
Cytochrome C oxidase assembly protein	Q76MV3	1.390	0.005
Acad9 protein	B1WC61	1.371	0.003
Thioredoxin, mitochondrial	P97615	1.312	0.049
Cytochrome c oxidase subunit 7A2, mitochondrial	P35171	1.311	0.000
Glutaredoxin-2, mitochondrial	Q6AXW1	1.243	0.031
NADPH--cytochrome P450 reductase	P00388	1.221	0.012
Mitochondrial import inner membrane translocase subunit TIM44	G3V640	1.220	0.046
Mitochondrial import inner membrane translocase subunit Tim21	Q5U2X7	1.219	0.020
NADH-cytochrome b5 reductase	G3V9S0	1.201	0.008
NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial	Q6PCU8	0.782	0.010
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 3	O08776	0.709	0.033
Peroxiredoxin 3	G3V7I0	0.703	0.018
Cytochrome b5	P00173	0.606	0.000
Hexokinase-2	P27881	0.573	0.003
Superoxide dismutase [Mn], mitochondrial	P07895	0.471	0.001
Peroxiredoxin 5, isoform CRA	A0A0G2JSS8	0.453	0.001
Glutathione peroxidase	M0RAM5	0.387	0.000
Adenylate kinase 4, mitochondrial	Q9WUS0	0.256	0.000

Table 3-7. Selected mitochondrial proteins of interest. Fold change= ratio of relative abundance of mean P14 result to mean Adult result. P- value is the result of a two- tailed Student's T- test comparing the two groups with the Bonferroni correction for multiple testing. Sorted by order of decreasing fold change. Positive values of fold change represent an increase in the 14- day heart. Adult group n= 7; P14 group n=8.

A subset of these proteins was identified that showed marked change in this analysis, and were also known to be of critical importance in both the handling of calcium during excitation and contraction of the heart, and involved in the process of cell death and injury or cardioprotection following ischaemia and reperfusion.

This subset was then examined across all four of the age groups; significant post-natal age-related variation was observed in a pattern maintained across the majority of proteins in this group (Figure 3-6).

3.5.4 Developmental changes in calcium and oxidative stress related proteins

Proteins including PLC, PKA, PKCε, LTCC, NCX, RyR and phospholamban all displayed significant age- related variation in a pattern which is similar to that the age-vulnerability relationship shown in Figure 1-17. Similar variation was shown in the analysis for catalase (Figure 3-7). Strikingly, however, other antioxidant enzymes, such as superoxide dismutase in both cytosolic and mitochondrial variants as well as glutathione peroxidase were up to 2- fold more abundant in the adult vs. pool; they showed an inverse pattern to that of catalase.

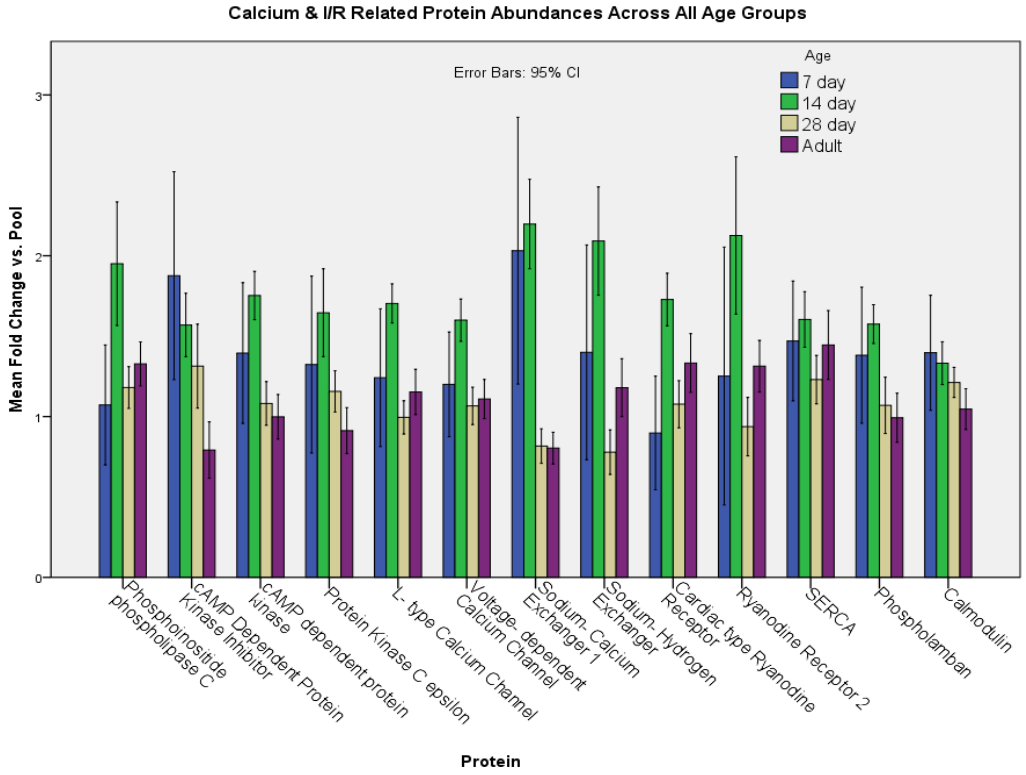
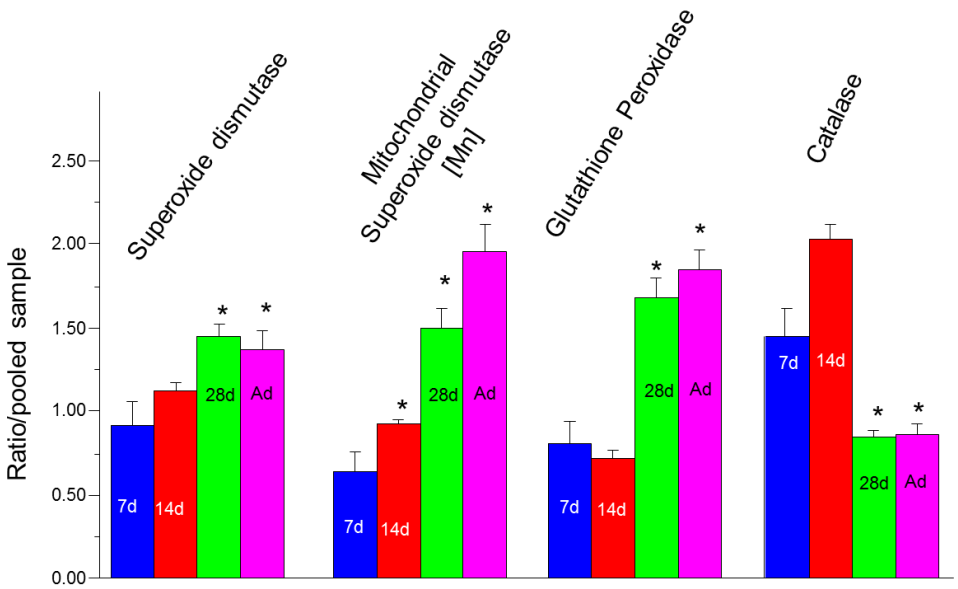


Figure 3-6 Selected proteins of importance to EC Coupling and I/R injury vs relative change in abundance in proteome of cardiac extracts of noted post-natal ages compared to poolError bars represent 95% confidence intervals. All results significant p< 0.05 (P14 vs adult) aside from for SERCA.



P- value is the result of a two- tailed Student's T- test comparing the two groups with the Bonferroni correction for multiple testing. Positive values of fold change refer to change vs pooled abundance. Adult group n= 7; P28 group n=8; P14 group n=8; P7 group n=4.Figure 3-7. Abundance of key anti-oxidant enzymes in proteome of

cardiac extracts from differing post-natal age groups showing age related variation in expression. Bars denote mean value. Asterisks represent significance p <0.05 vs pool. Error bars represent 95% confidence interval. P- value is the result of a two- tailed Student's T- test comparing the two groups with the Bonferroni correction for multiple testing. Ratio refers to abundance in sample vs pooled abundance. Adult group n= 7; P28 group n=8; P14 group n=8; P7 group n=4.

3.6 Pathway analysis

The proteomes were then analysed using Ingenuity Pathway Analysis (Qiagen, Germany) in order to assess the data without the drawback of subjectively looking at proteins or classes of interest. Figure 3-8 shows selected relevant signalling pathways compared to the expected abundance of all constituents of that pathway for both P14 and adult heart extracts colour coded by z-score for that difference. It shows that there are significant differences in the expression of members of key signalling and metabolic pathways. Key observations are that the adult shows an overrepresentation of oxidative phosphorylation, TCA cycle components and gluconeogenesis; there is also a mild increase in the level of β-AR pathway components. Conversely, the P14 group shows a relative increase in the level of PKA signalling as well as multiple pathways involved in the processes of development; however this group shows a marked underrepresentation of gluconeogenesis.

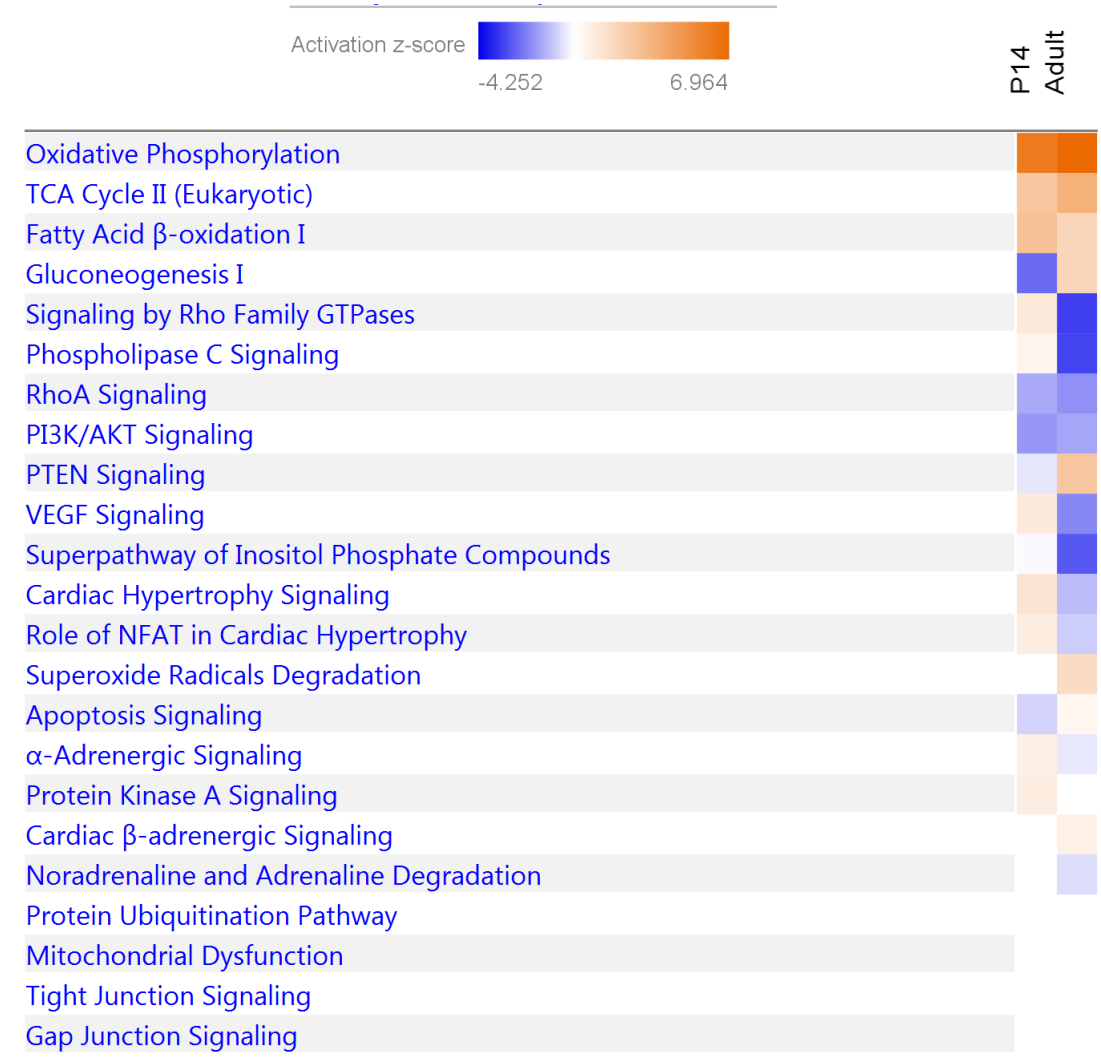


Figure 3-8 Mean expression of components of selected signalling pathways from P14 and adult age groups performed by automated pathway analysis. Colour intensity represents change from expected expression by z-score; orange colours are greater than expected and blue less than expected. Comparison to hypothesis of no significant difference between age groups. Group sizes- Adult n=7 P14 n=8. Output from Ingenuity Pathway Analysis, Qiagen, Germany.

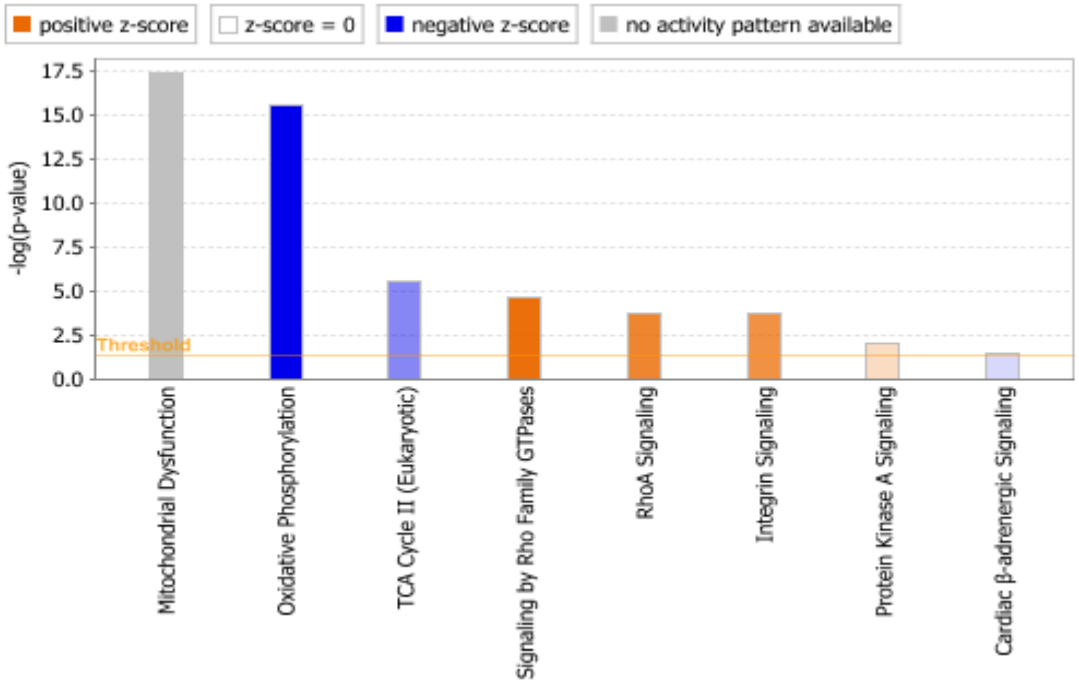


Figure 3-9 Changes in expression of selected relevant functional categories of biological importance comparing P14 day old and adult proteome. Significance value is shown on y- axis and direction of change by colour. Colour, representing z-score; negative scores represent relatively higher results in the adult, whilst positive in the P14. Significance testing performed by a 2-tailed T-test with correction for multiple testing. Group sizes- Adult n=7 P14 n=8. Output from Ingenuity Pathway Analysis, Qiagen, Germany.

The data were then assessed by functional category of protein rather than by signalling pathway in a comparative analysis between the P14 and adult groups; this analysis for selected categories of interest is shown in Figure 3-9. The changes in categories responsible for ATP production seen in Figure 3-8 are also noted here, but the most striking observation is that of the very significant representation of components labelled as involved in mitochondrial dysfunction. However, the analysis reports as no overall change in direction measured by z-score.

This category was then examined in closer detail (Figure 3-10). This shows diagrammatically the proteins identified in their schematic spatial location of origin. Then, a colour overlay is superimposed labelling those proteins by their relative abundance compared between the P14 and adult groups. This shows a mixed pattern; both complex I and complex IV constituents show age-dependent variation in abundance. However this pattern is not consistent across all constituents; whilst most show upregulation in the adult, others show the reverse.

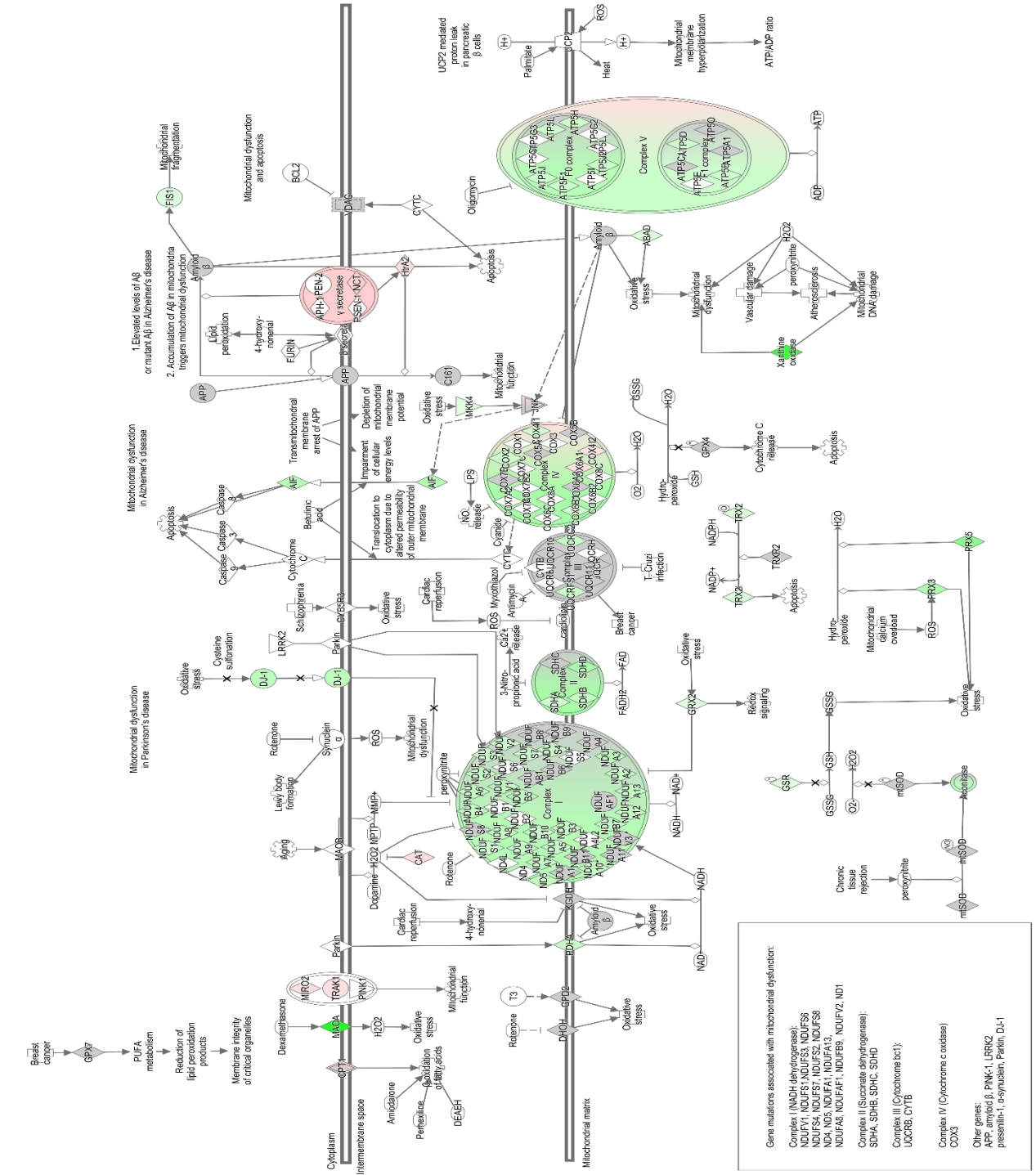


Figure 3-10 Detailed comparative view of known proteins involved in mitochondrial dysfunction with colour overlay denoting comparative changes between P14 and adult age groups. Expression logarithmic fold difference overlaid in colour; red= higher in P14, green= higher in adult. Group sizes- Adult n=7 P14 n=8. Output from Ingenuity Pathway Analysis, Qiagen, Germany.

3.7 Discussion

These findings are relatively broad but however the dataset that has been generated is novel. The rat is a well studied animal model in translational research as well as in the proteomic literature, and along with other mammals have also been used as a model of physiological human development. This relies upon the underpinning assumption that the patterns of gene/transcript/protein expression remain conserved across the species barrier. Recently, as large scale phenotyping techniques of genomics, transcriptomics and proteomics have become more established, many authors have attempted to validate or challenge these assertions. A recent study by Cardoso- Moreira & colleagues (Cardoso-Moreira, Halbert et al. 2019) studied the transcriptomes of multiple organs through key developmental timepoints across multiple species including the rat and the human. This is an extremely comprehensive study, validating the use of these species for comparative work through development. However, as far as could be found in searching the literature and public databases of available proteomes, to date no such similar work exists using a whole proteome approach. This work examined the changes in the unselected proteome with time through development at key milestones. Whilst many others have published on cardiac proteomics, these studies tend to be focused on the proteome of particular organelles in the rat (Ferreira, Vitorino et al. 2014), or pathological conditions- particularly in human studies- (O'Reilly, Lindsey et al. 2018), or have examined other organisms entirely. The most widely studied animal model in searching for other comparable work seems to be the mouse. Talman & colleagues (Talman, Teppo et al. 2018) have published a proteomic and metabolomic atlas of postnatal mouse heart development, studying molecular changes at a range of postnatal ages broadly similar to the milestones examined in this work. No similar published proteome exists for *rattus norvegicus*. Examining the PRIDE (PRoteomics IDEntifications) archive (Vizcaíno, Csordas et al. 2015) maintained by the EMBL-EBI (European Molecular Biology Laboratory- European Bioinformatics Institute), which is a world-leading repository of MS- based proteomic data, the archive lists 19 submitted cardiac proteomes. None of these survey the post-natal developmental

changes in the proteome, or address immaturity at all, and so the data presented in this chapter is novel at least amongst publicly available and acknowledged sources.

3.7.1 Summary of findings

Thousands of peptides and their parent proteins were identified in the proteomic comparative experiments. By automatically annotating them and filtering them by recognised subset categories, an attempt was made to avoid a selection bias in the analysis of changes in abundance between age groups.

Nevertheless, after this analysis it was necessary to focus on a tractable sample of these proteins within each set which were significantly and reliably altered between the groups being compared. This inevitably risks overlooking heretofore unknown proteins or correlations, but within the scope of the project is necessary.

The output from the experiments identified almost 7,000 unique proteins; however, Uniprot lists 29,951 proteins identified as members of the rat proteome. There are several reasons which might account for this difference. Firstly, the cardiac proteome is expected to be a subset of the whole. In the human, the cardiac proteome contains roughly 62% of the proteins expressed in the whole organism (Fagerberg, Hallström et al. 2014). However, the results here are proportionately lower than that ratio, albeit in the rat rather than in human; the other explanations are likely technical (e.g. protein extraction method) and are discussed in the limitations section; 8.2.2.

There were several main findings from the proteomic experiments comparing P14 to adult hearts.

- A significant trend towards overexpression of calcium homeostasis related proteins and selected signalling intermediaries in the P14 heart
- An overrepresentation of proteins localised to the mitochondria in the P14 heart
- A reduction in abundance of proteins involved in signalling via cAMP with increasing post-natal age
- A correlation between age and abundance of antioxidant protein expression
- Upregulation of TCA cycle and oxidative phosphorylation proteins in the adult vs the P14 heart

- Marked differences in many proteins regulating mitochondrial energy production, oxidative stress, and fission/fusion but split patterns for up/downregulation in the P14 vs the adult.

3.7.2 Changes in Ca^{2+} cycling proteins during development

The sodium-calcium exchanger (NCX) was 2.7- fold more abundant in the 14- day old vs. the adult samples. Other proteins intimately involved in calcium homeostasis were also identified as being significantly more abundant in the neonate vs. adult. The ryanodine receptor, which gates Ca^{2+} release from the SR was 1.6 fold more abundant. Phospholamban and CaMKII- targets for PKA and Epac signalling- were similarly upregulated, as well as others critical for excitation- contraction coupling.

Although the precise ultrastructural changes in the neonate as it develops towards adulthood are not precisely known, it is thought that the sources of intracellular calcium including and in particular the sarcoplasmic reticulum are relatively underdeveloped. The cardiomyocyte is therefore much more dependent upon extrusion of calcium as a means of terminating calcium, and so NCX, operating in the reverse mode, is relatively more important (Huang, Hove-Madsen et al. 2008). This explanation, however, would not account for the relative increase in RyR or the voltage- gated calcium channel; this may in an effort to maximise the release from the underdeveloped intracellular calcium stores; studies localising these proteins in developing hearts may be necessary to further account for this finding.

3.7.3 cAMP signalling proteins are downregulated with age

Of the 822 signalling proteins to be identified in the proteomic experiments, found prominently were those involved in cAMP linked signalling and the control of excitation- contraction coupling. cAMP dependent protein kinase- PKA- was found to be significantly upregulated over two- fold in the 14- day old heart as compared to the mature heart, as well as the (direct or indirect) Epac targets CaMKII, Phospholamban, and PKCε. Given the importance these targets have to cardioprotection, it is striking that the pattern of expression they display matches the vulnerability profile of developing heart to injury.

Proteins involved in shaping the spatial compartmentalisation of the cellular response to a signal were also found to be significantly upregulated in the developing heart. Caveolin- 3, an important scaffolding protein and the member of the caveolin family of most significance in cardiomyocytes (Lin, Hung et al. 2009), was 1.7 fold more abundant in the neonate than the adult comparator. Caveolin- 3 is known to interact significantly with and recruit the sodium calcium exchanger to caveolae; (Bossuyt, Taylor et al. 2002) caveolae in general act to produce foci for signalling proteins, recruiting regulatory and transducing elements to produce tight control of cellular responses. A- kinase anchor proteins (AKAP) similarly act to recruit PKA to these signalling complexes and are upregulated almost two- fold in the 14- day old compared to the adult.

Therefore it is unsurprising that in the environ of a developing heart, with a high level of ontological signalling and development occurring, that these specialist signalling structures should be relatively over-represented. This, however, is inferential evidence and should be confirmed by ultrastructural examination of the cardiomyocyte at each stage of development of the organism.

3.7.4 Age Related I/R Vulnerability correlated with Antioxidant activity

An unexpected pattern of variation was seen with several proteins involved in the response to oxidative stress; a key event in myocardial damage following ischaemia and reperfusion. Catalase, which catalyses the conversion of hydrogen peroxide, a key source of oxygen radicals, into water and molecular oxygen, seems to follow the same bell- shaped curve of vulnerability as the animal as a whole (Figure 1-17; Figure 3-7). However, glutathione peroxidase, which mainly reduces lipid hydroperoxides and superoxide dismutase (cytosolic and mitochondrial forms), which catalyses the conversion of superoxide radicals into molecular oxygen or hydrogen peroxide, showed a distinctly different pattern (Figure 3-7), rising steadily in abundance towards maturity. The reasons as to why the predominant antioxidant enzyme should shift as a heart develops towards adult are unclear. As the heart develops towards the mature phenotype, it shifts its primary metabolic substrate to fatty acids.

There may also be alterations in mitochondrial function or oxidative capacity and this might be an interesting topic for further study which would account for these observations.

3.7.5 Mitochondrial Dysfunction- related proteins vary with age

The output from the pathway analysis in the main confirmed the patterns observed from more manual inspection of proteins of interest. The most significant deviation from this was in the observation of marked variation in the expression of many mitochondrial proteins, in the domains of energy production- mostly observed in Complex I and Complex IV, as well as in assemblies responsible for fission and fusion dynamics, and some mitochondrial antioxidant activity. The mitochondria are complex multifunctional structures whose regulation is still not well understood, so to conclude that these observations must be responsible for other physiological phenomena is overly reductive; however it does suggest areas for investigation.

There are many marked areas of variation in the proteome as the heart develops towards its mature state. A full examination of each individual protein and signalling network as they change is beyond the scope of this work, but broadly surveying them does hint that the areas of Ca^{2+} homeostasis, signalling networks and mitochondrial biology play important roles in the physiological and pathophysiological features of ontology. The next chapter examines the differing response of the immature heart to ischaemia and reperfusion injury compared to the adult, and following chapters examine that response at the cardiomyocyte and mitochondrial level. The observations described here have suggested the mitochondria and the physiological role it plays in homeostasis and signalling may have bearing on the phenomena of I/R injury; subsequent chapters aim at exploring and delineating that relationship.

3.7.6 Relations with Prior Proteomic studies.

4 The cardioprotective efficacy of cAMP signalling pathways in immature and adult Langendorff perfused heart

4.1 Introduction

In Chapter 1, an effective intervention to protect the *ex vivo* perfused heart, from an injury due to global ischaemia and reperfusion, was described involving consecutive activation of PKA & PKC using isoprenaline and adenosine. However, it remains unclear as to whether this intervention can protect the immature heart.

This question arises because unlike the adult heart, the vulnerability of postnatal hearts to I/R changes rapidly over a timescale of days during development. It is thought to follow a triphasic pattern in rats studied in fundamental science models, with those of a postnatal age of 1 day, and also in the period between 14-21 days, being most resistant to I/R injury (Riva and Hearse 1991, Ostadalova, Ostadal et al. 1998, Modi and Suleiman 2004, Ostadal, Ostadalova et al. 2014).

Clinical research has also recapitulated this pattern of developmentally-related differences in cardiac vulnerability to I/R in patients undergoing open heart surgery (Imura, Caputo et al. 2001, Modi, Imura et al. 2003). The underlying mechanisms in clinical practice or in the experimental models are not well understood, but they may include developmental changes in caloric intake & lean mass (Ost'adalova and Babicky 2012, Ostadal, Ostadalova et al. 2014) and in Ca^{2+} mobilization and ROS production. This metabolic link could also involve cAMP/PKA signaling pathway, which is important in the regulation of cardiac Ca^{2+} cycling.

The mechanism by which this cardioprotective intervention through PKA & PKC is thought to occur is by pharmacologically mimicking the phenomenon of

temperature- preconditioning (Khaliulin, Parker et al. 2010). This depends upon activity of PKC for efficacy of the intervention (Khaliulin, Clarke et al. 2007).

That this intervention using isoprenaline is cardioprotective means that it necessarily involves the activation of both protein kinase A, as was evident at the time of its discovery, but also EPAC; this was a much less well understood cAMP sensor whilst the initial studies on ischaemic and temperature preconditioning were undertaken. It is then unclear as to which of these targets are sufficient and necessary for the observed cardioprotective effect.

In the physiological setting, β- AR activation should activate PKA and EPAC to a consistent degree based upon their respective responses to cAMP. Khaliulin & colleagues (Khaliulin, Bond et al. 2017) used cell permeable engineered cAMP analogues as molecular probes, capable of selectively activating PKA and EPAC. This method allows isolation of the physiological and pathophysiological roles of these effectors, and so an examination of the contributions of these pathways to protection from ischaemia & reperfusion injury.

4.2 Aims

Whilst the cardioprotective efficacy of adrenergic receptor stimulation as a means of modulating cardioprotection in the heart is well established in adult heart (Khaliulin, Parker et al. 2010), little is known about its efficacy in immature heart. However, as previously discussed (Chapter 1), the physiology of the immature heart at all levels, from gross structure and function through receptor pharmacology and calcium homeostasis differs enormously from the adult phenotype. There is therefore insufficient cause to *a priori* assume that effective interventions in the adult will necessarily operate identically in the immature heart.

The proposed work in this chapter was designed to address the hypothesis that simultaneous PKA & Epac stimulation or consecutive Iso/Aden stimulation confer protection in immature as well as in adult heart. To tackle this hypothesis, the following aims were addressed:

1. to establish the physiological function of isoprenaline in the immature heart as compared to the adult. This was measured by assessment of contractile function in a Langendorff perfusion model in a constant pressure mode, which then further allowed surrogate assessment of coronary vascular resistance as a proxy of coronary flow rate with constant perfusion pressure.

2. to assess if and to what extent consecutive perfusion with isoprenaline and adenosine is cardioprotective against a global ischaemic & reperfusion injury in the immature heart and to what extent.

3. To move downstream from the β-adrenergic receptor and use synthetic cell permeable cAMP analogues to directly stimulate PKA and EPAC. This will allow assessment of the protective effect in a similar model of global ischaemia and reperfusion used for the second aim, in both adult and immature rat heart.

4.3 Methods

Hearts, obtained from male Wistar rats, were perfused in the Langendorff mode as described in Chapter 2. Briefly, however, animals were killed by cervical dislocation after stunning by concussion. Hearts were then rapidly excised, immersed and rinsed in chilled buffer. The aorta was identified and secured onto a cannula through which warmed & oxygenated Krebs- Henseleit (KH) buffer was being pumped.

In order for reasonable cross experimental comparison, exclusion criteria were used for all Langendorff experiments such that hearts were excluded if cannulation took more than two minutes to establishment of the equilibrium conditions, if regular rhythmic contraction did not become established or if there was an obvious dysrhythmia.

4.3.1 Experimental Protocols

The following protocols involving ex vivo heart perfusion were adopted:

Protocol 1: The initial set examined the physiological effects of isoprenaline perfusion in adult and P14 hearts. Controls in this protocol were perfused in the same manner but with standard KH buffer instead of buffer with the addition of isoprenaline.

Protocol 2: the second, using the same hearts that had been treated with isoprenaline- the cardioprotective benefits of isoprenaline and sequential adenosine perfusion with a comparison between the effects in adult and P14 hearts. For both Protocols 1 & 2, the intervention arm hearts- those receiving a drug treatment- had a 30 minute equilibration period, followed by 2 minutes perfusion with 5nM isoprenaline in KH buffer with 5 minutes perfusion with KH buffer and 300nM adenosine immediately following (Protocol 2 only). Similar to the control arm, they then had a 30 minute period of global ischaemia and a 120 minute period of reperfusion (Figure 4-1).

Control experiments in this protocol used perfusion with standard KH buffer for 7 minutes instead of 2 minutes isoprenaline and 5 minutes adenosine, followed by 30 minutes global ischaemia induced by cessation of flow, followed by a 120 minute reperfusion period. These were performed by constant pressure perfusion, with an intraventricular balloon connected to a pressure transducer in order to provide continual measurements of left ventricular pressure.

For these experiments, a second pressure transducer in line with the perfusion circuit was used to provide feedback control of the perfusion pressure. This was maintained at 60 mmHg in the immature group and 80 mmHg in the adult group.

Throughout these protocols, functional measurements (LVDP & Flow Rate) were made continuously and for the purposes of analysis exported at 5 minute intervals. Coronary effluent was collected at the end of equilibration and at 5 minute intervals throughout the reperfusion period.

Protocol 3: examined the cardioprotective effects of perfusion with cAMP analogues in both adults and P14 hearts, in the same model of ischaemia & reperfusion injury as the first set. This protocol is also shown graphically in Figure 4-1.

The control hearts in this group also had a 30 minute equilibration period, followed by a 30 min period of global ischaemia, and subsequently a 120 minute reperfusion period; intervention hearts were given a 5 minute period of perfusion with a cAMP analogue (8-Br/ 6-Bnz/ CPT), followed by a 5 minute washout period and then a 30 minute period of ischaemia with a 120 minute period of reperfusion.

These hearts were perfused at a set flow rate. Experience with the constant pressure group above was that that technique led to a high experimental failure rate due to variability in the resistance that could be generated to flow especially in the immature heart; in some circumstances it was difficult to generate sufficient pressure to meet criteria at a reasonable range of perfusate flow rates and so these

experiments were carried out with a more traditional constant flow arrangement. Therefore, the flow rates were set based on the resultant initial flow rate from the earlier constant pressure experiments, which produced results consistent with other published data; 10 ml/min for the adult hearts and 4 ml/min for the immature. Consequently, in this group LV and CP pressures were not measured, and the outcome measures were solely histological and biochemical.

For all protocols, global ischaemia was produced by stopping the pump which delivered the perfusate. During this period, the hearts were immersed in KH buffer maintained a 37° C by a water jacket connected to the main reservoir and thermostat.

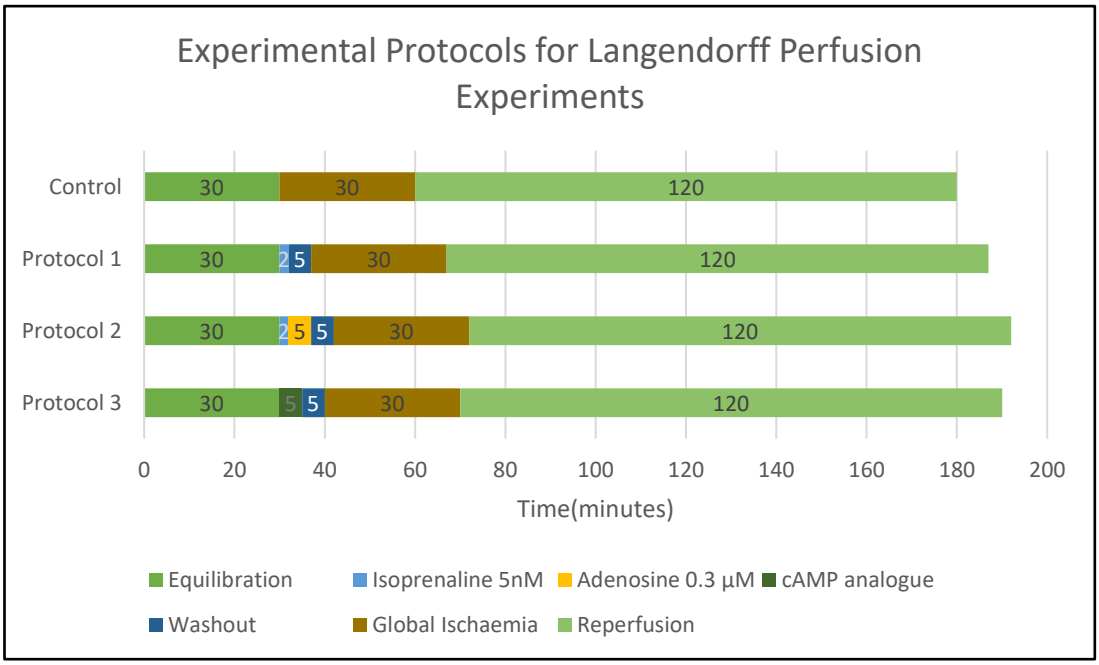


Figure 4-1. Graphical representations of the experimental protocols used for ex vivo heart perfusion for Protocols 1, 2 & 3showing the durations of each phase of the protocol and the drugs added to the perfusate at each stage.Numbers in each bar represent the duration of that stage, in minutes. Control refers to the controls used in each of Protocols 1, 2 & 3.

4.3.2 Exclusion Criteria

Hearts were rejected and not included in further analysis based upon the following exclusion criteria; these are essentially all surrogate markers for inadequate *ex vivo* perfusion of the heart.

1. If the cannulation time (time from excision from the animal until flow restored *ex vivo*) was greater than 2 minutes. This represented an excessive as well as uncontrolled and non-analysed degree of ischaemic stress to a heart and so was presumed to render a result uninterpretable and certainly non-comparable to other hearts cannulated more rapidly.
2. If the heart had multiple (3 or greater) attempts at cannulation. Multiple attempts at cannulation were associated with damage both to the aorta through vessel wall injury, as well as to structures such as the root & coronary vessels as well as the aortic valve. Such attempts were likely to fail later in the experimental protocol due to a failure of adequate perfusion and so were recognised and excluded early in each experimental run.
3. If the insertion of the LV pressure balloon caused distortion which was impeding contraction by external inspection or if insertion caused damage. The insertion of the LV balloon is necessarily traumatic. However, occasionally the particular combination of balloon insertion and balloon pressure inside the LV cavity, as well torque from the catheter associated with the balloon caused a distortion to the anatomy of the heart being studied such that adequate perfusion and contractile function could not be obtained. In these cases, in experimental series requiring measurements of LV pressure, the heart was excluded.
4. If the heart rate did not stabilise at >200 beats per minute after the 30 minute equilibration period. Failure of normalisation of the heart rate may have a range of causes in the perfused heart- incorrect buffer, exclusion of a certain coronary artery from perfusion by occlusion/ aortic dissection/ embolization, complete failure of cannulation, aortic valve inadequacy; for instance. Failure of these issues to be simply corrected meant that the heart was not suitable to be included.

5. If more than one episode of ventricular arrhythmia was encountered during the stabilisation period. Physically evident or electrically detected ventricular dysrhythmia or arrhythmia following cannulation prior to experimental ischaemia was also felt to reflect a failure of the isolation process. Again, this had a variety of causes from incorrect electrolyte concentrations within the perfusate through to mechanical issues with cannulation and inadvertent aortic valve rupture leading to LV overload. However for all these processes the effect of episodes of ventricular fibrillation, for instance, would render the heart incomparable to hearts which had not suffered a similar injury and so these were excluded from further experimentation.

A total of 19 hearts fell into these exclusion categories and were not included in the analysis. However, all hearts which had a successful run through their stabilisation period and started the experimental phases were included in subsequent analysis. The numbers of hearts analysed from each age group for these experiments are detailed in Table 4-1 for experimental protocols 1 & 2. Table 4-2 shows the numbers of hearts used for experiments in Protocol 3 and the associated controls, subdivided by the cAMP analogue used.

Age Group	*Control*	*Protocol 1*	*Protocol 2*
Adult	8	7	7
P28	5		6
P14	5	6	6

Table 4-1 Numbers of hearts included in analysis of ex vivo *perfusion experiments for each age group studied vs experimental protocol as described in 4.3.1. Protocols 1 & 2 and associated controls only. P28 hearts used for non-functional experiments only.*

Age group	*Control*	*8-Br*	*6-Bnz*	*CPT*
Adult	6	6	6	6
P14 (30 min ischaemia)	6	6	6	6
P14 (50 min ischaemia)	6	6	6	6

Table 4-2 Numbers of hearts included in analysis of ex vivo *perfusion experiments for each age group studied in Protocol 3 and associated controls.*

Measurements were made of coronary flow rate (CFR) at the set perfusion pressure (so coronary flow rate was a function of coronary vascular resistance), of heart rate derived from the LV pressure trace, and of the LV developed pressure. The rate-pressure product was then calculated as the arithmetic product of the developed pressure and heart rate measurements.

4.4 Results

4.4.1 The effect of isoprenaline on Cardiac Function in adult and developing heart

4.4.1.1 The Effects on Coronary Flow Rate

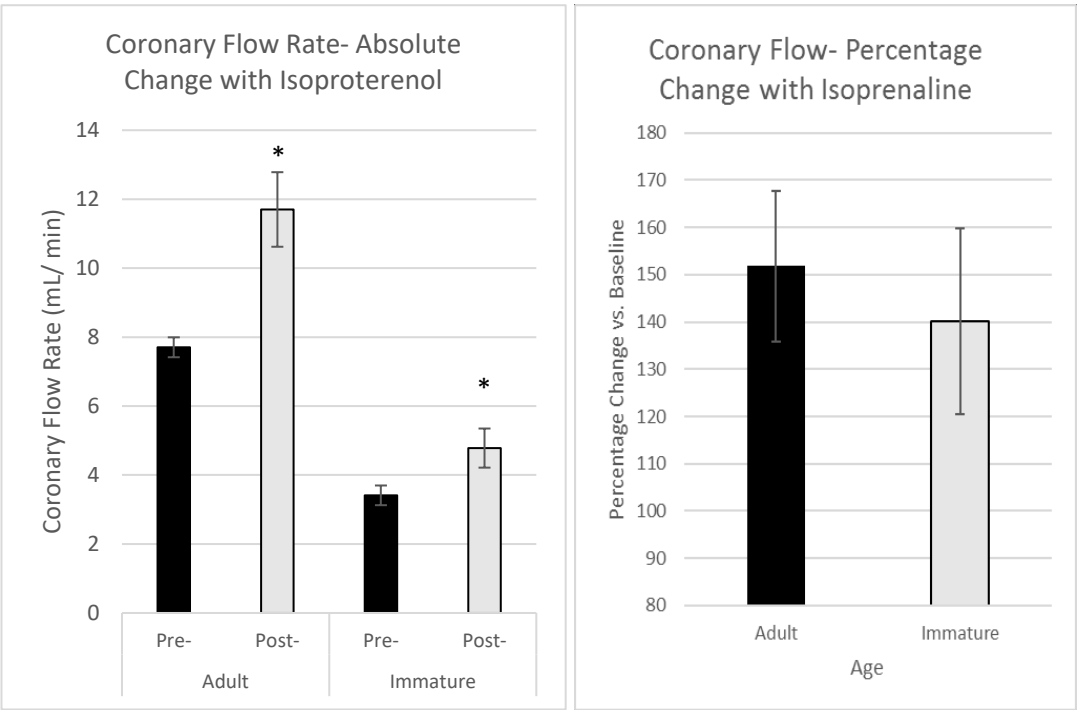


Figure 4-2. The effect of isoprenaline upon coronary flow rate. Left panel; Absolute coronary flow prior to and following addition of 5 nM isoprenaline to perfusate in P14 (n=6) and adult (n=7) male perfused Wistar rat hearts. Right panel; proportionate changes relative to control for each age group. Error bars represent 95% confidence interval. Asterisk- p< 0.05 vs pre- intervention result. Significance testing by Student's T-test.

The CFR was significantly increased in both groups following administration of 5 nM isoprenaline (Figure 4-2). The adult group increased from an average value of 7.7 ml/min to 11.7; the P14 group showed an increase from 3.4 ml/min to 4.8. Both increased around the same proportion; there was no significant (intergroup) difference in the percentage increase (151.9% vs baseline in the adult, 140.2% in the 14- day old animals.

4.4.1.2 *The effects on Developed Pressure*

Similar to the results for CFR, developed left ventricular pressure increased significantly in both the adult (mean from 51.2 mmHg to 87.2) and the 14- day old group (mean from 33.3 mmHg to 55.8) (Figure 4-3). There was no difference between the groups in the proportionate increase in developed pressure, however. The adult group showed an increase of 70.2% compared to an increase of 67.2% in the neonatal group.

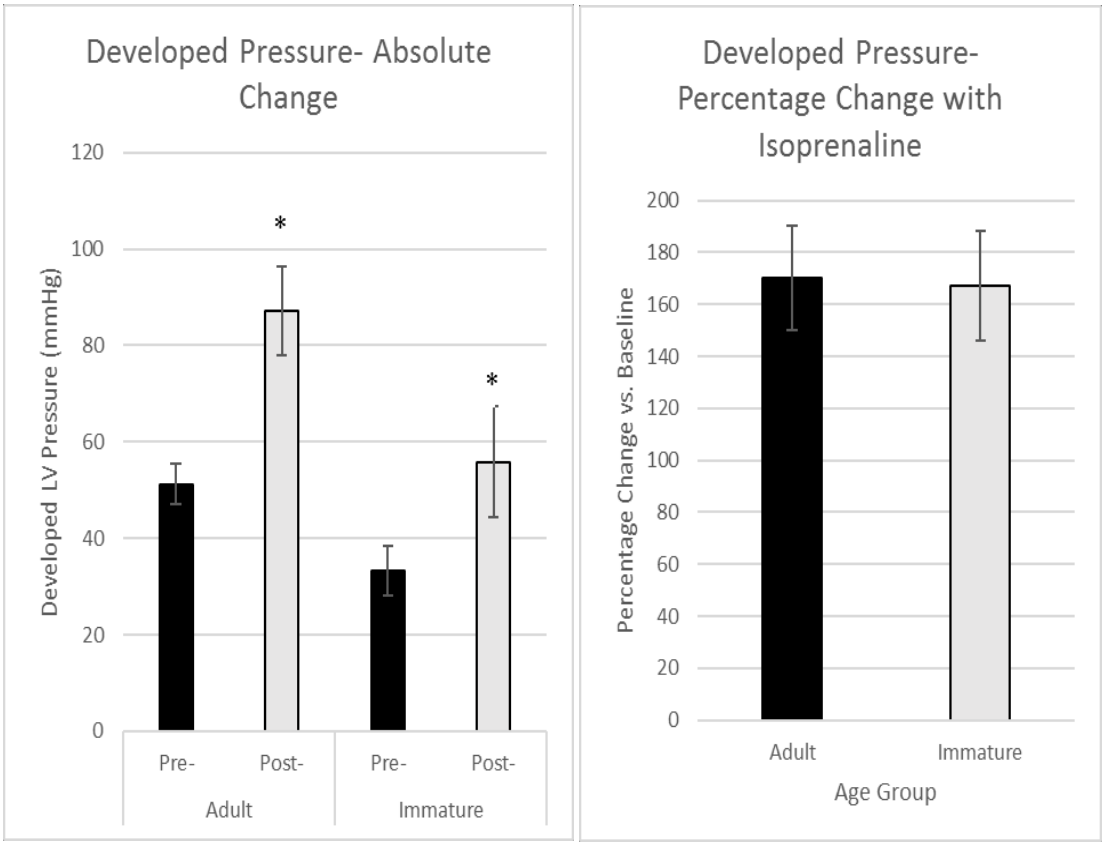


Figure 4-3. The effect of isoprenaline upon developed pressure in the isolated perfused heart. Left panel; Absolute pressure developed in the left ventricle prior to and following addition of 5 nM isoprenaline to perfusate in P14 (n=6) and adult (n=7) male perfused Wistar rat hearts. Right panel; proportionate changes relative to control for each age group. Error bars represent 95% confidence interval. Asterisk- p< 0.05 vs pre- intervention result. Significance testing by Student's T-test.

4.4.1.3 *The effects on Rate Pressure Product (RPP)*

The rate- pressure product is the arithmetic product of the developed pressure and the heart rate at that time point and is considered to be a surrogate marker of cardiac work. In both the P14 group and in the adults a significant rise in RPP was observed after treatment with isoprenaline (208.2 % from 9591 mmHg min^{-1} to 19,970 in neonates, 160.8% from 14,793 mmHg min^{-1} to 23,790 in adults, Figure 4-4). However, a significant intergroup difference was also observed in the response to isoprenaline. Whilst the RPP in both groups increased markedly and significantly after exposure to isoprenaline, the increase in RPP in the P14 group was 78.6% more than in the adult.

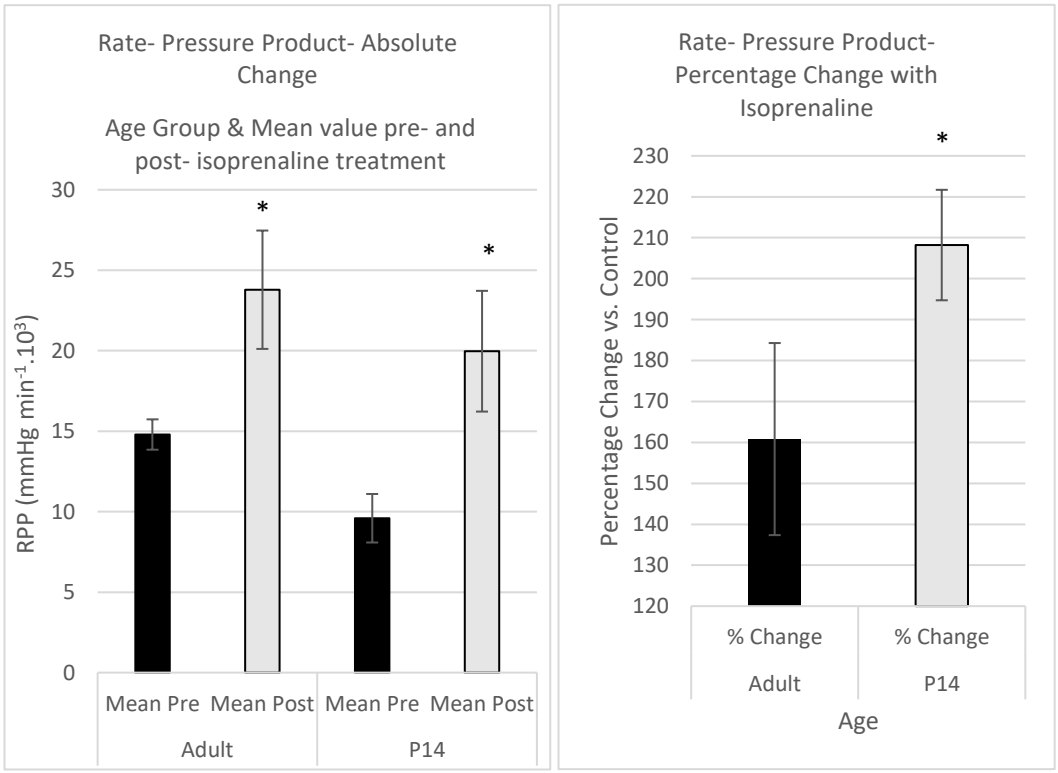


Figure 4-4.The effect on Rate- Pressure product of isoprenaline. Left panel; Absolute rate- pressure product measured in the left ventricle prior to and following addition of 5 nM isoprenaline to perfusate in P14 (n=6) and adult (n=7) male perfused Wistar rat hearts. Right panel; proportionate changes relative to control for each age group. Error bars represent 95% confidence interval. Asterisk- p< 0.05 vs pre- intervention result. Significance testing by Student's T-test.

4.4.2 The cardioprotective efficacy of Iso/Aden treatement in Developing & Adult Hearts

As the hearts used in this work were the same as those in section 4.4.1 for Protocol 1, the numbers used for each protocol and age group as well as the success rates are the same. That is- 37 hearts were perfused and had a technically successful run through the experimental or control protocol, with 19 others excluded by the experimental criteria. Of those included, 15 were of the adult age group (7 experimental, 8 control), and 11 14- day old (6 experimental, 5 control). An additional group of 11 28 day- old (6 experimental & 5 control) was added *post hoc* as an additional comparator which was only assessed for LDH release and infarct size. The criteria for inclusion or exclusion remained the same as in section 4.4.1. A typical pressure- time trace for an experiment is shown in Figure 4-5.

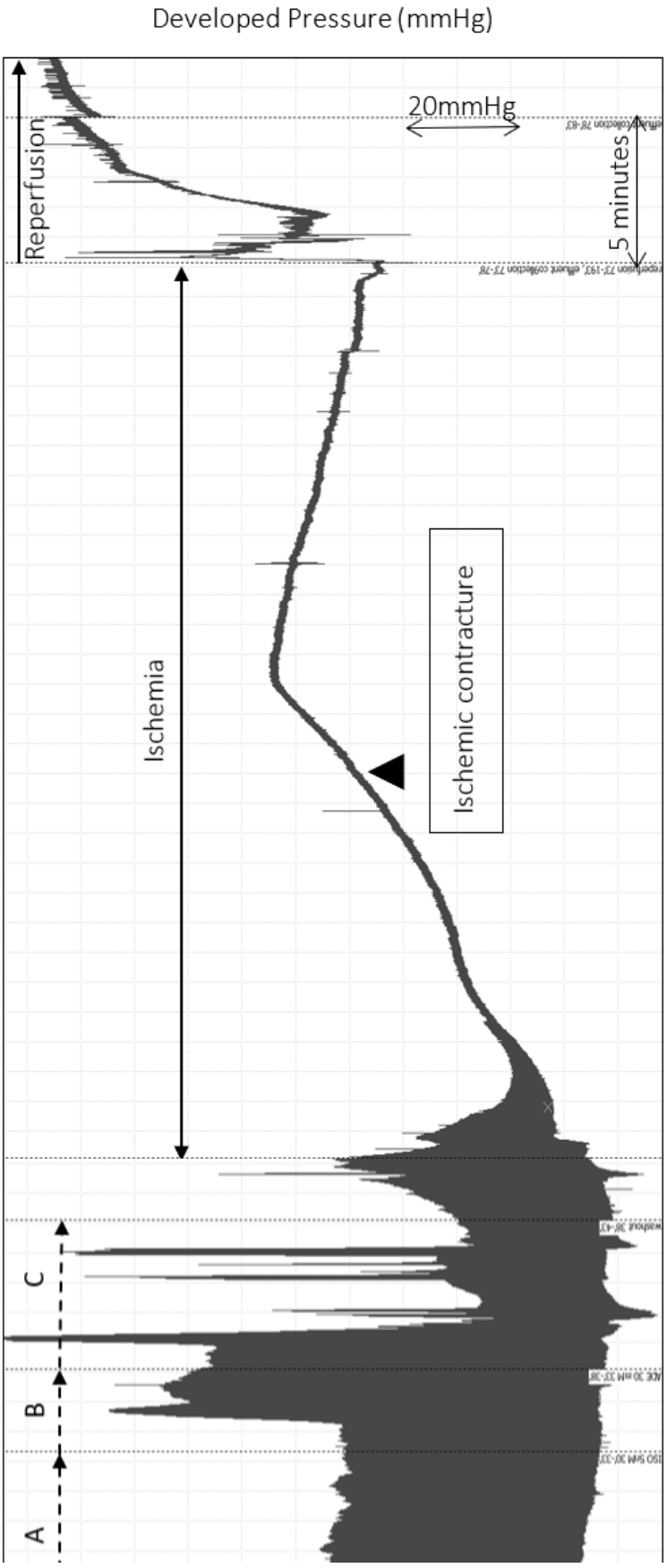


Figure 4-5 Representative LV pressure- time trace from adult heart perfused with Isoprenaline then Adenosine. Y- axis; LV pressure/mmHg; X-axis; time/min.Letters represent differing experimental conditions. A- Equilibration; B- Isoprenaline perfusion; C-Adenosine Perfusion

4.4.2.1 Functional Recovery

The summary of recovery at the end of the experimental protocols is shown in Figure 4-7, whilst Figure 4-6 shows the recovery vs time after reperfusion for both age groups and experimental groups. There was no significant difference between control and intervention arms in the 14- day old hearts, but there was a significant difference between the two arms in the adult group either expressed as a comparison between control and intervention group RPPs at the end of the experiment or as proportion of function retained after the experimental protocol.

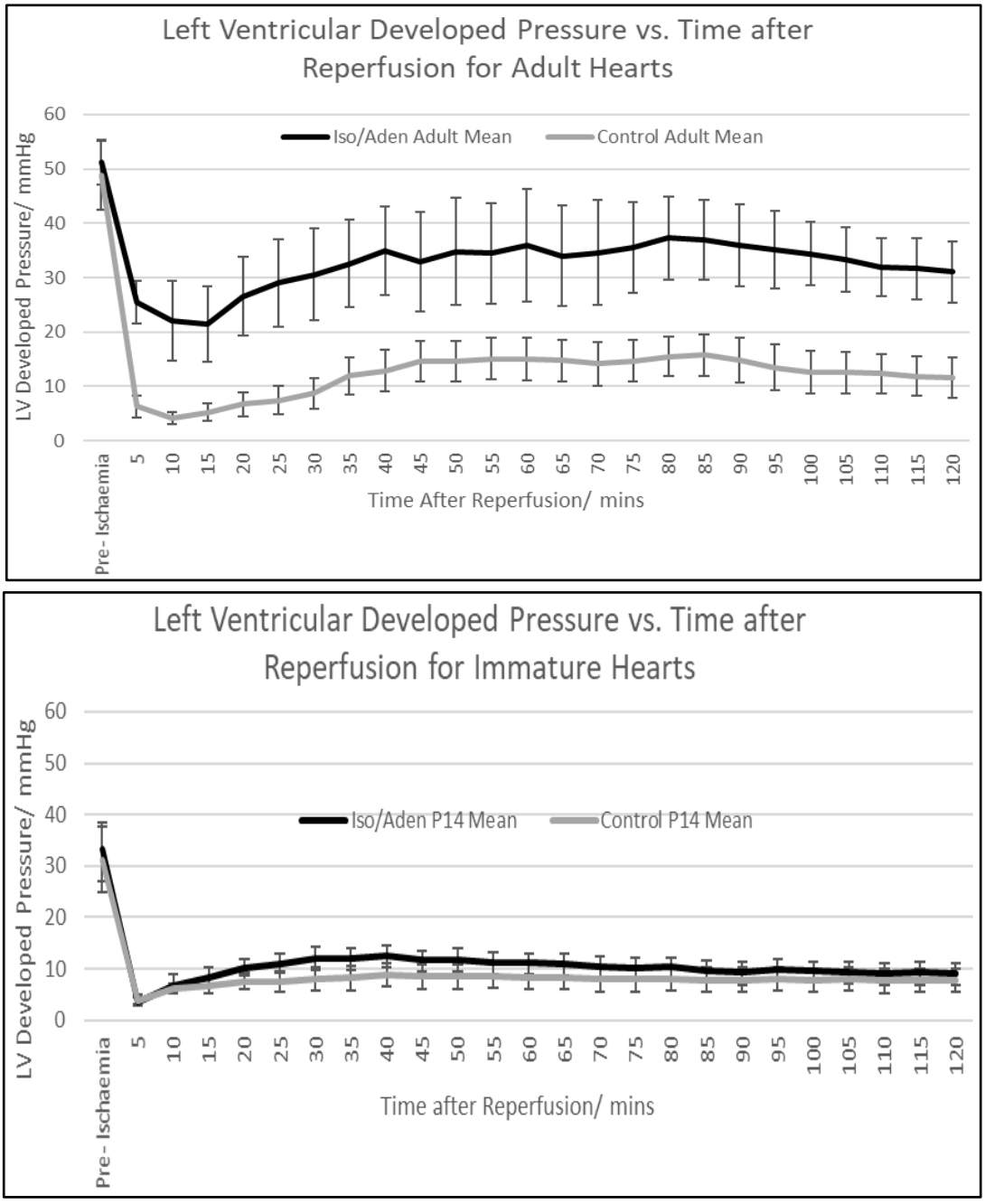


Figure 4-6 Mean LV Developed pressure vs. Time after reperfusion for adult and immature (P14) hearts given isoprenaline/adenosine treatment or control. Error bars represent Mean ±SE. Adult group n= 7 intervention, n= 8 control, P14 n=6 intervention, n= 5 control. Top panel adult hearts; Bottom panel- immature (P14) hearts.

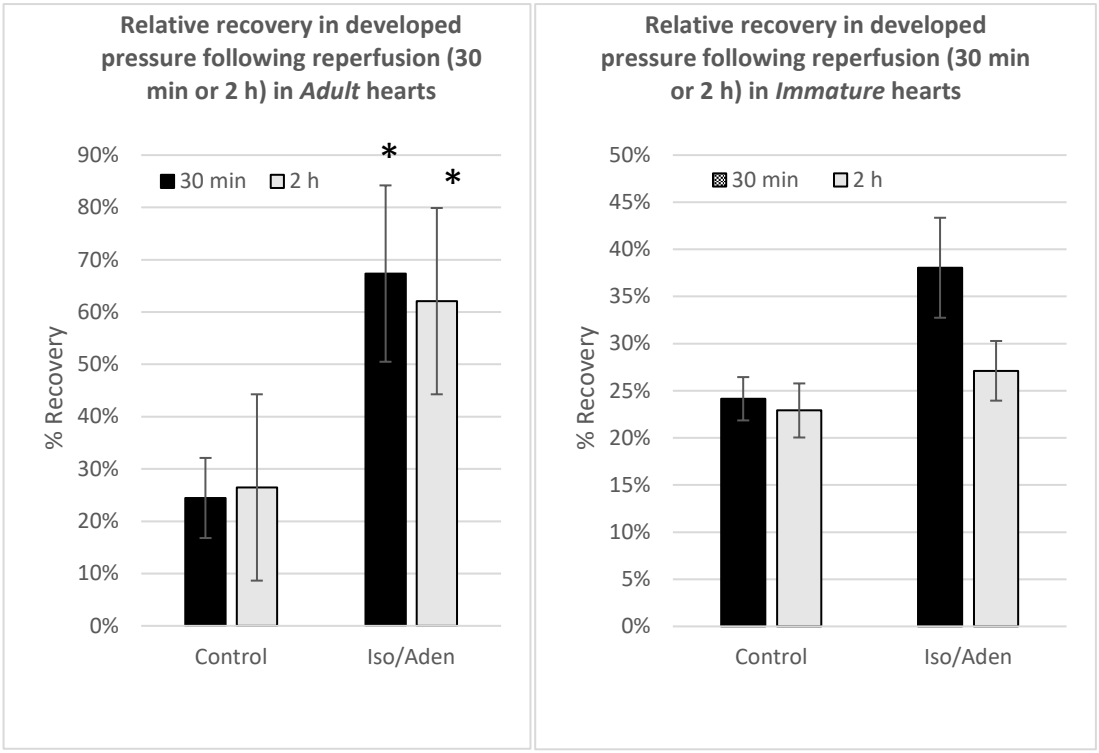


Figure 4-7 Proportionate haemodynamic outcomes for adult (n=7 iso/aden, n= 8 control) and immature (P14, n=6 iso/aden n=5 control) hearts following recovery from ischaemia/ reperfusion injury comparing control to those treated with isoprenaline/ adenosine. Plotted as proportion (Mean ±SE) of developed pressure from equilibration period attained at 30 minutes and 2 hours of reperfusion.Control = no intervention, Iso/ Aden= consecutive isoprenaline/ adenosine treatment.. Asterisk; p<0.05 vs corresponding control. Significance testing by Student's T-test.

4.4.2.2 Effect on LDH Activity in effluent

For LDH activity (Figure 4-8, Figure 4-9, Figure 4-10), all age groups showed a reduction in LDH released into coronary effluent between the control and intervention arms which appeared to be significant. In the adult group, this release of LDH seemed to go on beyond the period over which fractions were collected but in the 14- and 28- day old groups the activity fell towards the end of the collection period. For the adult, the maximum difference occurred at 25 minutes following reperfusion where the mean difference was 55.5 mU/ml vs 102.7 showing that the release of LDH in hearts exposed to I/A protocol was 54% of that in the controls. For the 14- day old hearts, the peak difference occurred at 20 minutes following reperfusion, with a mean LDH activity of 44.6 mU/ml for the I/A group compared to 117.3 in the control group, so the LDH release in the treatment group for 14- day olds was only 38% of that in controls. In the 28- day old group, maximal difference

occurred at 15 minutes into reperfusion where the control group had an LDH activity of 86.5 mU/ml whereas the interventions had 39.2 mU/ml/, 45.3% of the untreated. However, for this group, by the end of the coronary effluent collection period, there was no discernible difference in LDH activity unlike in the adult and 14- day groups.

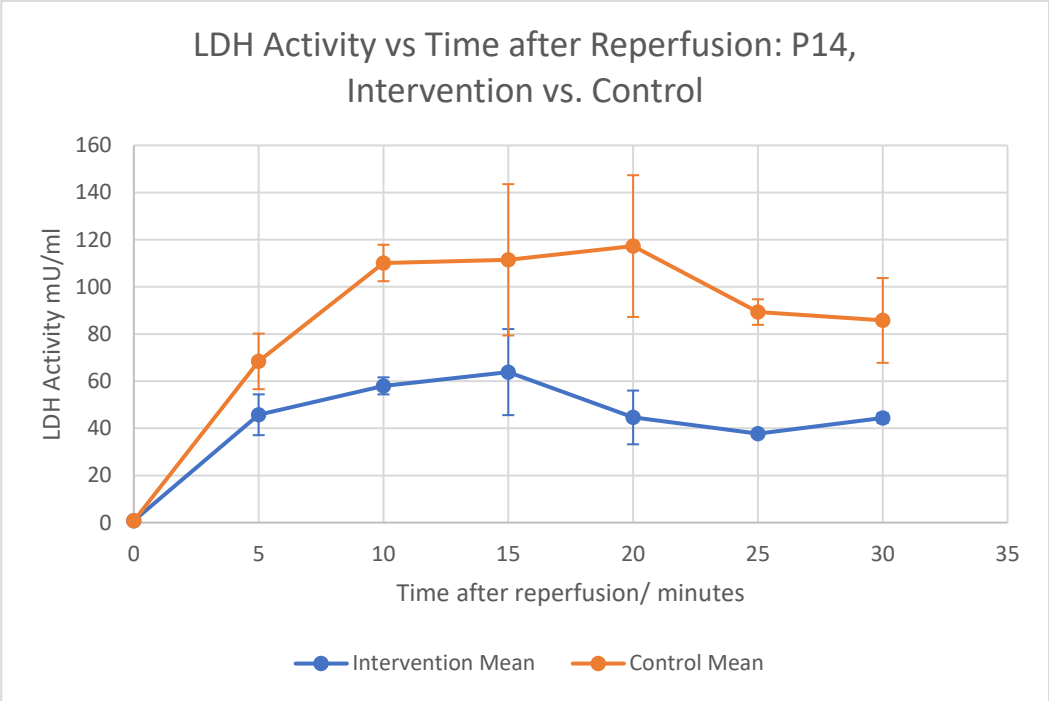


Figure 4-8. LDH activity in coronary effluent vs time after start of reperfusion for intervention (isoprenaline & adenosine treatment) and control experiments for 14 day old hearts exposed to 30 min global ischaemia. Time= 0 is from a pre-ischaemia fraction. Data presented as Mean ±SE (n=6 intervention, 5 control).

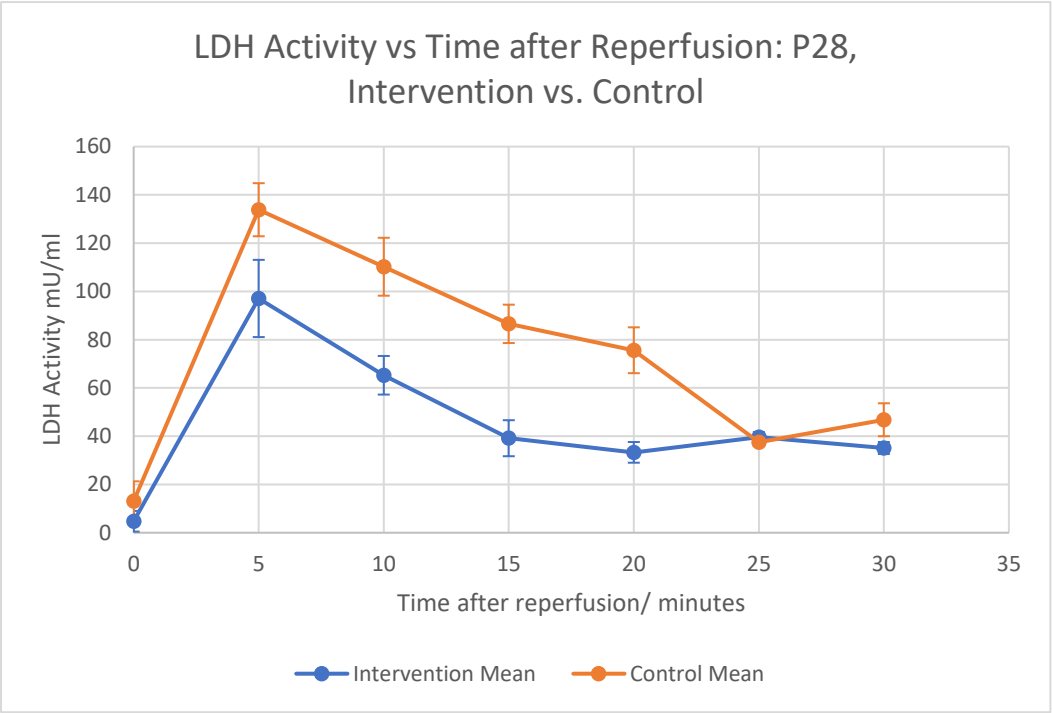


Figure 4-9. LDH activity in coronary effluent vs time after start of reperfusion for intervention (isoprenaline & adenosine treatment) and control experiments for 28 day old hearts exposed to 30 min global ischaemia.Time= 0 is from a pre-ischaemia fraction. Error bars represent Mean ±SE (n=6 intervention, 5 control).

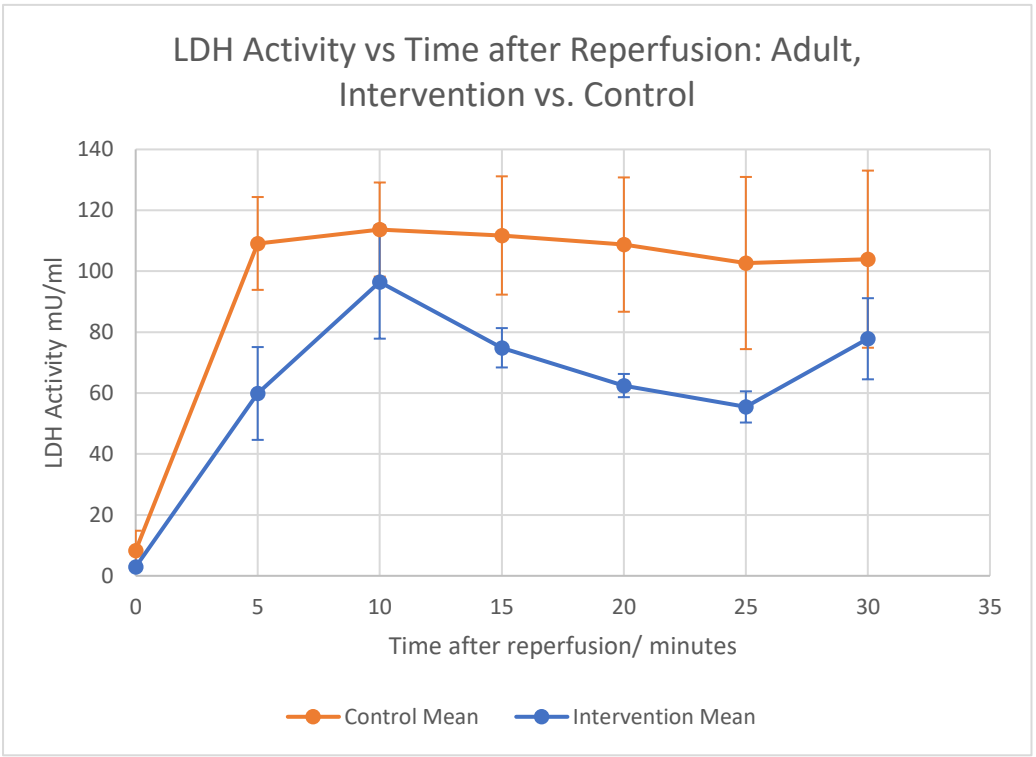


Figure 4-10. LDH activity in coronary effluent vs time after start of reperfusion for intervention (isoprenaline & adenosine treatment) and control experiments for adult hearts exposed to 30 min global ischaemia.Time= 0 is from a pre-ischaemia fraction. Error bars represent Mean ±SE (n= 7 intervention, 8 control).

4.4.2.3 *Effect on Infarct Size*

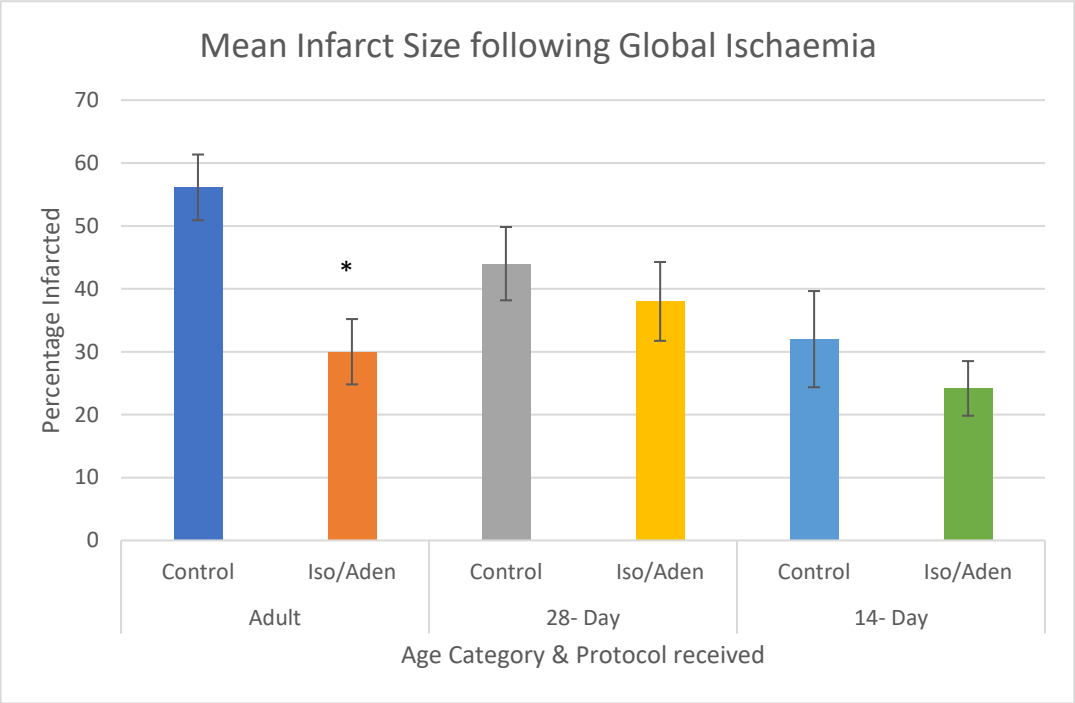


*Figure 4-11. The effect of isoprenaline/ adenosine perfusion upon infarct size following ischaemia/ reperfusion injury. Infarct size expressed as cross sectional area proportionate infarct. Intervention here means consecutive isoprenaline/ adenosine treatment. Error bars represent Mean ±SE; *= p< 0.05 vs same aged control tested by Student's T-test. Group sizes- adult n= 7 intervention, 8 control, P28 n=6 intervention, 5 control, P14 n=6 intervention, 5 control.*

There were also significant differences in the area of myocardial tissue infarcted after exposure to the two protocols (Figure 4-11). In the adult, the difference was significant ($p< 0.05$) with an infarcted area of 56.1% for the controls vs 29.9% in intervention group. For the other two groups, the results showed a similar trend but did not reach statistical significance in either the 28- day old group (44.1% vs 38.5%) or the 14- day old group (32.0% vs 24.2%). The intra- group control comparison does show an increasing vulnerability to infarction with increasing age after 14 post- natal days.

4.4.3 Cardioprotective effects of cAMP analogues

For the set of experiments used for assessment of the effect of perfusion with cAMP analogues (Protocol 3), 6 hearts per drug per age group were included. That is 6 adults and 6 14 day old rat pups for each experiment with 8-Br, 6-Bnz, and CPT, with a further 6 in each of those age groups for controls. *Post- hoc*, another group of P14 experiments were carried out with a longer ischaemic duration of 50 minutes in order that the magnitude of infarct in the control matched that of the adult controls, to increase the magnitude of injury to the heart and enable cross age group comparison of the effect of the drugs independent of the magnitude of injury.

For these experiments, an intraventricular balloon was not used due to the experience of the variation and excess failure its use had caused in the isoprenaline ± adenosine experiments. Further, creatine kinase (CK) activity was measured instead of lactate dehydrogenase (LDH) due to differing availability of reagents when these experiments were carried out.

4.4.3.1 The effects on CK Activity in the effluent

Figure 4-12, Figure 4-13 and Figure 4-14 show the activity of creatine kinase in the coronary effluent taken at 5 minutes intervals from adult and P14 hearts, perfused with cAMP analogues prior to ischaemia, normalised to the coronary flow rates used in those experiments. Figure 4-15 shows an estimate of the area under each of the activity-time curve as a collective measure of CK release in the period following reperfusion.

The adult hearts, Figure 4-12, show that CK release occurs rapidly in the control group, peaking in the 5- minute fraction. This is in contrast to the activity from those hearts perfused with either 8-Br, 6-Bnz or CPT prior to ischaemia. All of those groups had a delayed peak, and a reduced total release vs. control. CPT and 6-Bnz had an indistinguishable effect from one another, but 8-Br produced the greatest reduction in CK release at all time points and in cumulative activity over the 30 minute period studied.

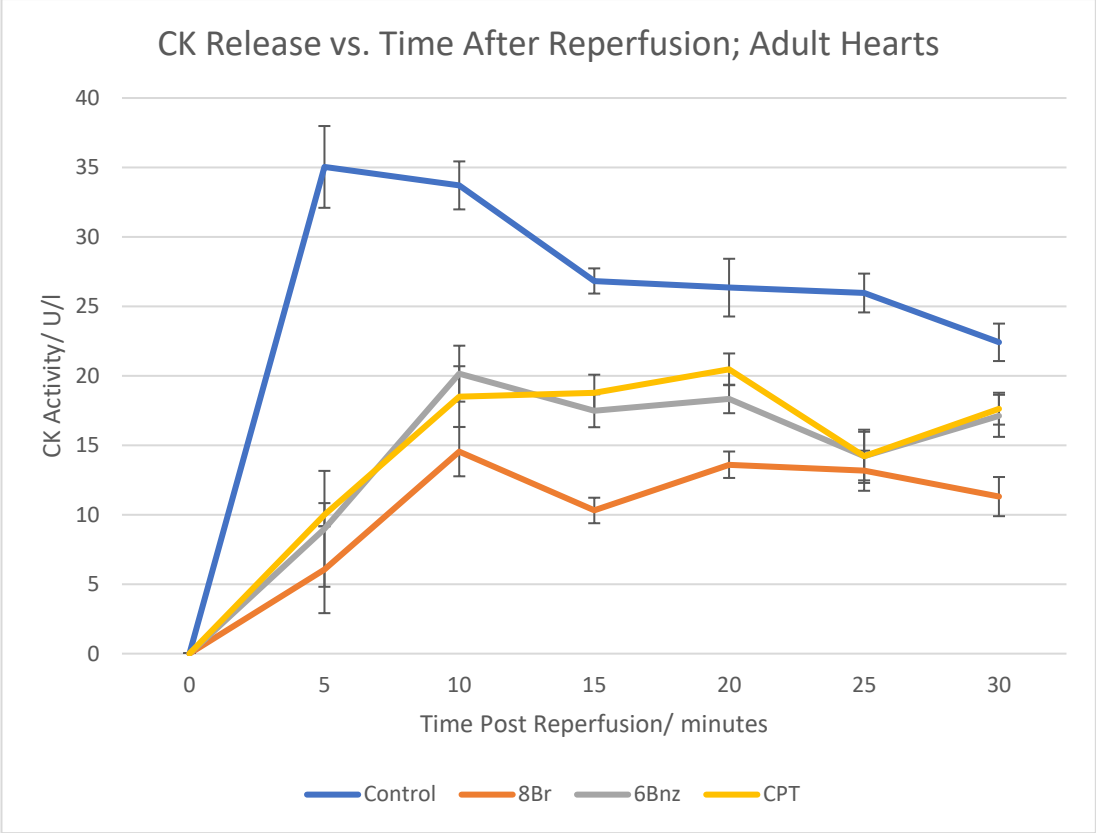


Figure 4-12 Activity of creatine kinase in coronary effluent from adult hearts in prior to (t=0) and following reperfusion after 30 minutes of global ischaemia measured at 5 minute intervals with preceding perfusion with cAMP analogues vs control. Normalised to coronary flow rate. Error bars represent Mean ±SE; n=6 per group.

The data for P14 hearts exposed to 30 minutes of global ischaemia is shown in Figure 4-13. In these hearts, the control group demonstrated a peak activity level of 58.6% that of the adult; this peak was arrived at significantly later than in the adult, in the 15 minute fraction rather than in the 5 minute sample. In this group, however, none of the cAMP analogues significantly reduced the overall CK activity in the period studied. The ratio of areas under the activity-time curve for the control in this group vs. that in the adult was 57.1%; so the degree of injury sustained as measured by the CK release was significantly lower.

However, for the group of P14 hearts exposed to 50 minutes global ischaemia, Figure 4-14, this ratio was 97.06%; so the longer period of ischaemia resulted in a more directly comparable degree of injury. In this group, there was a marked reduction in CK activity with all of the cAMP analogues used; this was most marked with 8-Br but

a reduction in AUC was seen with both CPT and 6-Bnz as well. This was statistically significant for 6-Bnz but not for CPT.

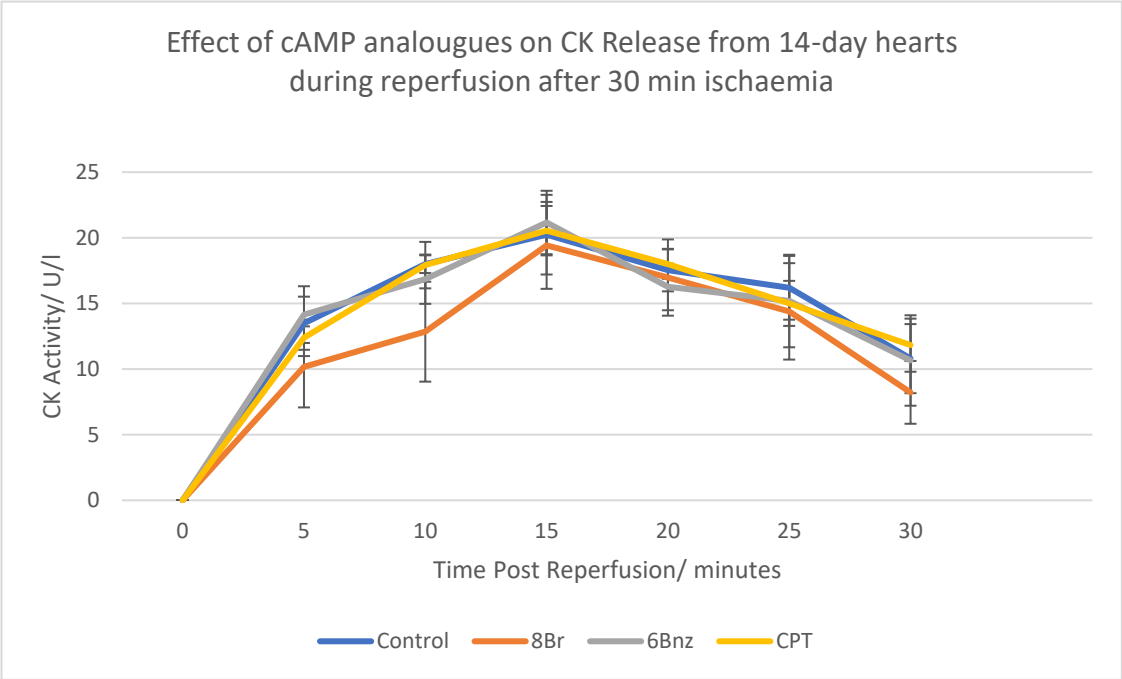


Figure 4-13 Activity of creatine kinase in coronary effluent from 14- day old hearts in prior to (t=0) and following reperfusion after 30 minutes of global ischaemia measured at 5 minute intervals with preceding perfusion with cAMP analogues vs control. Normalised to coronary flow rate. Error bars represent Mean ±SE; n=6 per group.

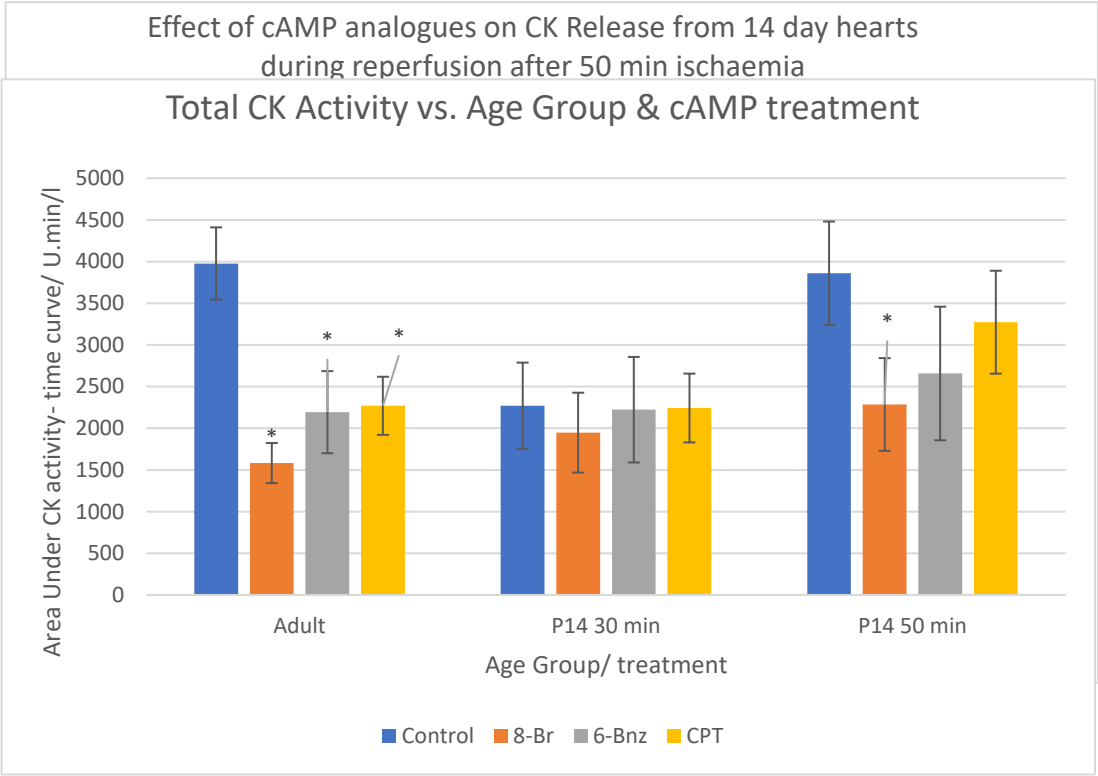


Figure 4-14 Activity of creatine kinase in coronary effluent from 14- day old hearts in prior to (t=0) and following reperfusion after 50 minutes of global ischaemia measured at 5 minute intervals with preceding perfusion with cAMP analogues vs control. Normalised to coronary flow rate. Error bars represent Mean ±SE; n=6 per group.

*Figure 4-15 Total CK activity in coronary effluent following reperfusion in hearts exposed to ischaemia/ reperfusion injury with or without perfusion with a cAMP analogue. The P14 hearts were divided into a 30- minute and a 50 minute ischaemia group. N=6 per analogue per age group. Total activity taken as estimated area under activity-time curves summed by trapezoidal addition of mean values for each time point. Bars represent mean AUC ± SE; * = p<0.05 vs same age group control. Statistical testing by Student's T-test.*

4.4.3.2 *The effect of cAMP analogues on Infarction size*

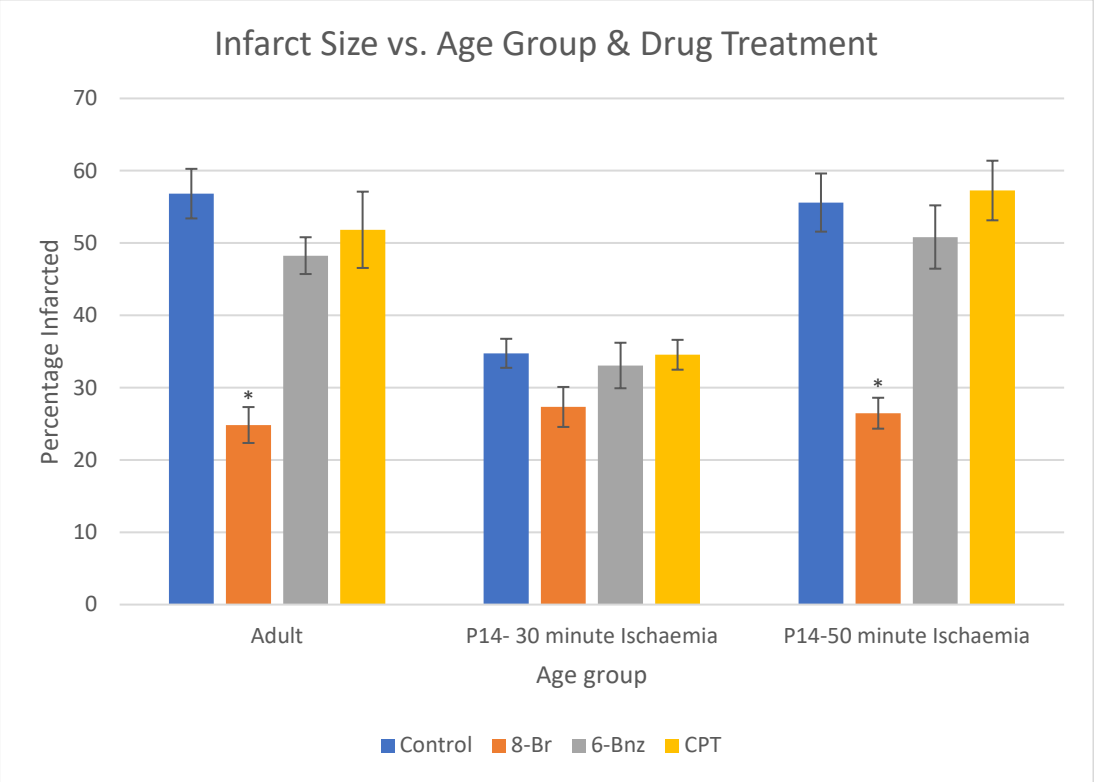


*Figure 4-16 The effect of cAMP analogue perfusion upon infarct size following ischaemia/ reperfusion injury. Infarct size expressed as cross sectional area proportionate infarct. Mean ±SE; *= p<0.05 vs. same age group control by Student's T-test. The P14 hearts were divided into a 30- minute and a 50 minute ischaemia group. N=6 per analogue per age group.*

The resulting infarct sizes for all the hearts treated with cAMP analogues, Protocol 3, are shown in Figure 4-16. In adult hearts, a 30- minute ischaemic injury in the control group produced a mean infarct area of 56.8%. This is consistent with the degree of injury sustained in adult hearts in the earlier (4.4.2.3) experiments performed with constant pressure perfusion.

For the drug treated hearts, perfusion with 8-Br, the non-selective cAMP analogue, produced a marked and significant reduction of the area infarcted, to a mean of 24.8% representing an infarct 43.7% the size in the control group. Neither perfusion with 6-Bnz to stimulate PKA, nor CPT to increase EPAC activity were associated with a significant reduction in infarct size in the adult hearts.

The group of P14 hearts subjected to an ischaemic injury of 30 minutes in length, showed an average infarct size of 34.75%. None of the cAMP analogues in this group produced a statistically significant reduction in the area infarcted.

However, when the ischaemic time was increased to 50 minutes, the infarcted area increased to 55.6%, comparable to the adult hearts given a 30 minute cessation of perfusion. In this group, addition of 8-Br prior to ischaemia did produce a reduction in the infarct size- to 26.4%, a significant difference. Again, perfusion with 6-Bnz or CPT did not meaningfully reduce the infarct size; so in no group was perfusion with a either of these agents sufficient to reduce the magnitude of injury by this measure.

4.5 Discussion

The key findings from this work are that firstly isoprenaline is at least as effective inotrope in the immature heart as in the adult. It increases developed pressure and coronary flow to the same degree as in the adult heart, whilst rate-pressure product increases to an even greater extent. Secondly, it has been shown that sequential perfusion with isoprenaline & adenosine is an effective cardioprotective intervention in the adult, but not in the immature heart. Finally, stimulation of the β adrenergic signalling pathways (cAMP/PKA/Epac) seems to produce cardioprotection in the adult and immature heart.

4.5.1 Isoprenaline -induced inotropic effect is more marked in immature compared to adult heart.

The perfusion data presented here demonstrates, broadly, the expected physiological response of stimulation of the β-AR by isoprenaline. Developed pressure and coronary flow rates both significantly increased after addition of isoprenaline, as did the rate- pressure product.

Isoprenaline is an agonist at the β-AR with no selectivity between the β_1, β_2, or β_3 isoforms of the receptor (Ahlquist 1976). Therefore, it would be expected to have predominantly β_1 mediated actions upon the heart, which canonically lead to G-protein mediated activation of adenylate cyclase and raised intracellular cAMP levels. This in turn leads phosphorylation of components of the E-C coupling machinery including Ca^{2+}-cycling proteins and Troponin I and thus enhancing the overall contraction-relaxation cycle of the heart. The resultant increase in intracellular Ca^{2+} increases the force of contraction and also stimulates energy production by activating mitochondrial dehydrogenases (Halestrap & Denton 1980).

There is a significant increase in developed pressure in both the 14- day and adult groups, consistent with the expected cardiac response to β-AR stimulation. For the 14- day old group, there was a 67% increase vs a 70% increase in the adult- an insignificant difference within the accuracy of the measurement techniques used.

This confirms that isoprenaline is functioning as a positive inotrope at the cardiac adrenoreceptors in both age groups.

The results for RPP are at first glance fairly similar. However, there is a marked difference in the proportionate response to isoprenaline, in contrast to that for DP. The RPP for 14-day old animals increased 79% more than it did in the adults. This variation implies profound differences in the response to elevation in cAMP in the adult compared to the neonate- the HR elevation in response to isoprenaline was on average 35.7% greater in the neonate than in the adult. Typically, the neonate will increase cardiac output through increases in HR due to a multifactorial limitation in increasing contractility –so the observation here that, albeit in a non- physiological model- potent stimulation of PKA and Epac simultaneously can achieve a significantly higher increase in cardiac work in the neonate compared to the adult is striking. However, these observations occur at differing sites; an effect upon developed pressure occurs directly at the ventricular cardiomyocyte, whereas an organised increase in heart rate would be driven by effects at the sinoatrial node.

In the controlled pressure Langendorff system, there is a constant and steady flow of coronary perfusate. This allows a simple model to be conceived of for coronary flow rates; the coronary perfusion pressure (CPP- the set pressure in the perfusion system) should be proportional to the product of the coronary flow rate and the coronary vascular resistance (CVR). Or, the coronary flow rate is proportional to CPP/CVR. If the CPP is externally controlled, then the only influence on the varying CFR is the coronary vascular resistance.

There are two main possibilities to account for the observations regarding coronary blood flow. The most likely explanation is that isoprenaline, as a potent agonist at cardiac β adrenoreceptors, directly causes potent vasodilatation and thus an increase in CFR in the context of a constant CPP.

Another possibility is intrinsic alteration of the CVR. Under normal physiological conditions, the coronary vascular resistance is autoregulated; this is under local

control (from a variety of factors including hypoperfusion and wall shear stress) with wider autonomic influence also contributing. In the Langendorff system the extra cardiac influences are removed but it is possible that the intrinsic mechanisms of autoregulation remain intact. However, in human models of autoregulation, traumatic and surgical injury to an organ can attenuate or abolish the effect. It cannot be excluded that in the context on increasing cardiac work through the use of a potent positive inotrope such as isoprenaline that local hypoperfusion contributes to vasodilation rather it being solely a direct pharmacological effect.

4.5.2 Functional effects of isoprenaline & adenosine perfusion

These experiments showed a significant protective effect for consecutive administration of isoprenaline and adenosine across in adults. This reaffirms the previous observation (Khaliulin, Parker et al. 2010) that this provides a cardioprotective intervention in the well- studied adult heart. Thus, it reaffirms that cAMP modulation can precondition the heart against ischaemic insults. This intervention, however, is dependent upon an unselective PKA/ Epac agonist in isoprenaline. It is not clear to what extent each of the parallel signalling pathways that extends from PKA & Epac is responsible for the observed preconditioning, nor whether this changes in the differently aged groups which were studied. Others have reported that in Langendorff systems at least, Epac stimulation in adult rat hearts does not produce preconditioning or cardioprotection (Duquesnes, Derangeon et al. 2010).

It is interesting that despite signs of biochemical and histological protection (4.5.3.1, 4.5.3.2) that no functional benefit was observed in the 14- day old group, and only a trend to significance with a large degree of variability in the 28- day old group. These contradictory findings may represent an increased propensity for the phenomenon of ‘myocardial stunning’ following ischaemia in the developing age groups, or it may be that experimental variability obscured an effect.

4.5.3 Protective effects of Consecutive PKA & PKC activation

4.5.3.1 Biochemical Markers of Injury

All three experimental age groups showed a reduction in LDH release and activity, as a measure of injury, in their intervention arms compared to control. This was significant at almost all time points. Comparing age groups shows that peak injury occurs a little later- at 10 minutes rather than 5 minutes- in the 14- day old age group compared to the 28- day or adult. The youngest group also demonstrates slightly less LDH release into the coronary effluent than in the adult. That said, differences in the heart weight were not explicitly controlled for; rather the differential coronary flow rate provided by the constant pressure control perfusion method was relied upon in order to normalise perfusate flow. However, in each of these groups the intervention arm had significantly less LDH release than control, so on an intra- group comparison basis it seems reasonable to conclude that the intervention was protective. This is confirmed with the histological data showing a reduction in infarct size in each group vs. control.

4.5.3.2 Histological Parameters

Measurement of infarct size by TTC staining demonstrated a significant reduction in infarct area in all three groups. Considering just the control arms, the pattern of vulnerability observed in Figure 1-17 seems to be maintained; the adult is the most vulnerable to ischaemia and reperfusion injury, with the 14- day old relatively resistant (32% infarction in the neonate compared to 56.2% in the adult). This matches previous experience in our group, showing a bell-shaped distribution to ischaemic vulnerability. However, others (Ostadalova, Ostadal et al. 1998) have shown a bimodal distribution, with an earlier (prior to the 7th post- natal day) peak in resistance as well as that found around the 14th day post- natal.

The adult group showed a significant protective effect from the isoprenaline and adenosine treatment with an infarct size in the intervention arm 53.3% of that in the controls; although the other two groups showed some relative benefit, it was both smaller in magnitude and did not reach significance.

Thus on a number of measures this treatment of consecutive activation of PKA and PKC by isoprenaline and adenosine perfusion seems to be markedly cardioprotective in the adult male Wistar rat. However, this protective effect, although there were hints that it may be present in the neonatal groups, was not so marked and so did not reach significance on any measure other than LDH release. This may reflect the fact that these groups, were more resistant at baseline to the ischaemic stimulus, and so a protective effect would be somewhat masked by this innate resistance. It may be that if the length of time the global ischaemia had been applied for had been extended from 30 minutes in the neonatal groups to produce an identical degree of ischaemic damage as the adult in the existing protocol that a more marked protective effect may have been seen.

There are known to be significant metabolic differences between adult and immature heart which may be relevant. Previous work in our group has established significant variation in glycogen content; Figure 4-17 from (Lewis, Szobi et al. 2018).

4.5.3.3 Glycogen levels correlate with vulnerability

Unlike adult hearts, the immature heart has a lower level of glycogen content which is depleted relatively quickly during index ischemia. Thus, it is likely that isoprenaline/adenosine pre-treatment, by depleting the relatively lower levels of glycogen at an early stage during index ischemia, blunted any expected potential benefits from this intervention, probably due to a significant dysfunction of ATP-dependent ion channels (Kalogeris, Baines et al. 2012). Although the role of HKII association and dissociation to mitochondria and the link to glycogen content has been addressed, little is known about this in immature hearts.

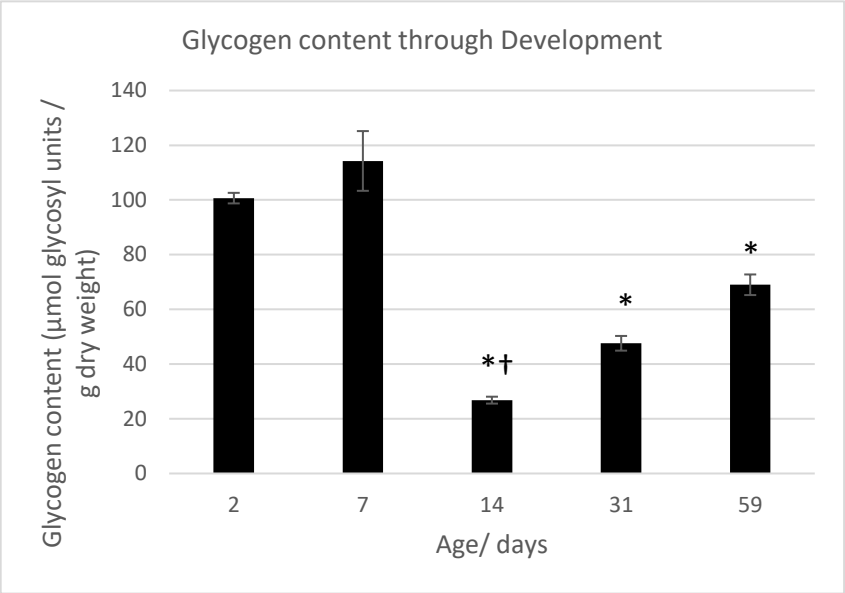


*Figure 4-17 Glycogen levels in hearts during development. Data analysed by 1-way ANOVA with Fisher's PLSD as a post-hoc test of significance. *=p<0.01 vs. both 2 days of age and 7 days of age. †= $p<0.001$ vs adult hearts. Data points represent mean ± standard error. From (Lewis, Szobi et al. 2018).*

Cardiac protection through temperature preconditioning has been well described, and shown to be necessarily dependent upon PKA and PKCε signalling for effective transduction of the

protective effect which involves inhibition of mitochondrial permeability transition pore (MPTP) opening on reperfusion. (Khaliulin, Clarke et al. 2007, Khaliulin, Parker et al. 2010).

Adenosine causes association of PKC, HSP 90 and a mitochondrial import complex TOM70 ultimately resulting in import of PKCε to the mitochondrion, and pharmacological inhibition of this translocation abolishes the cardioprotective action of PKCε (Yang, Sun et al. 2012). The ultimate mitochondrial targets of PKCε are unclear, although some have suggested interactions with the MPTP (Baines, Song et al. 2003, Budas and Mochly-Rosen 2007). The pore is more likely to open in ischaemic conditions- that is, high intracellular calcium, acidosis, and depletion of ATP, and opening of the pore in the context of ischaemia is associated with the onset of necrosis or apoptosis. Other possible targets have been proposed to be cytochrome

c (subunit IV in particular) and the ATP sensitive mitochondrial K^+ channel. Thus these targets give plausible mechanisms to account for the role of adenosine linked PKC in cardioprotection from ischaemia and reperfusion injury.

Isoprenaline acting via β-AR results in an increase in intracellular cAMP, leading to activation of PKA & Epac with downstream signal transduction. Ischaemic and other forms of preconditioning have for some time been known to attenuate cAMP levels during a sustained ischaemic injury (Lochner, Genade et al. 1999), with an increased cAMP level during an insult following a preconditioning stimulus being associated with a protective effect. This effect is observed not only with β-AR stimulation via isoprenaline but also using other means of modulating cAMP levels- via cAMP modulators, phosphodiesterase inhibitors, or direct activation of adenylate cyclase (Sanada, Asanuma et al. 2004).

There are multiple intracellular events that follow from a rise in cAMP levels within the cardiomyocyte; PKA and Epac act as sensors for the cAMP levels and so the cardioprotective effect of this rise must be mediated by their downstream targets. These targets are known to include a number of proteins involved in calcium homeostasis and excitation- contraction coupling, including phospholamban, the ryanodine receptor, and, via Epac induced Phospholipase C activity, PKCε. Thus, there is the possibility for cross- talk between stimuli causing increased cAMP activity via a β-AR, and, through Epac activity, cardioprotective mitochondrial PKCε activity.

That the cardioprotective effect is synergistic between these two pathways, with PKC and PKA/Epac as their primary effectors, acting in parallel suggests that it is the downstream targets that are significant for cardioprotection rather than any intermediary acting in common for both pathways. So the next set of experiments, involving the study of cAMP analogues stimulating PKA or Epac or both are important in demonstrating the relative effects.

4.5.4 The cardioprotective effects of PKA & Epac activation in whole heart perfusion

4.5.4.1 Direct and indirect activation of PKA & EPAC & cardiac function

In this study, I have not shown evidence of the functional effects of PKA and EPAC stimulation using the described cAMP analogues on intact perfused heart. These experiments were omitted because of the difficulty in reliably obtaining pressure-time traces from the immature perfused heart. Their effects upon the contractility of individual isolated cardiomyocytes are discussed in Chapter 6.

However, as discussed in Section 4.5.2, the functional effects may be important mechanistically through depletion of metabolic substrates including glycogen. It is therefore important to know to what extent perfusion with the cAMP analogues used in these experiments replicates the inotropic and chronotropic effects of isoprenaline perfusion when considering their cardioprotective properties. This has been demonstrated by others, at least in the adult heart. Khaliulin & colleagues, (Khaliulin, Bond et al. 2017) in our group performed a similar set of experiments, on perfused adult rat heart, using the same agents at the same concentrations. They show a 2.5 fold increase in RPP after perfusion with 5 µM 8-Br, for 5 minutes followed by a washout period where the RPP returns to baseline. This is broadly similar in magnitude to that shown in these experiments for the effects of isoprenaline in Figure 4-4. They further show that using the selective agonists 6-Bnz and CPT for activity at PKA and EPAC respectively, each agent individually does not produce an equivalent result. Only when administered in combination does the cardiac work increase to the same level as that seen with 8-Br, and isoprenaline in the results presented here. Given that the functional effects of isoprenaline on the immature heart are similar to those in the adult, it is reasonable to expect that the cAMP analogues would be have similarly in the P14 hearts.

4.5.4.2 *Combined stimulation of PKA & Epac provides maximal protection against injury in the adult heart*

The experiments described here show that PKA and Epac stimulation produces cardioprotection in the *ex vivo* perfused adult rat heart. Consistent with previous findings, (Khaliulin, Bond et al. 2017) simultaneous stimulation of both parallel signalling pathways appears to be necessary for maximal cardioprotection in this model of ischaemia & reperfusion injury; stimulation of either PKA or EPAC alone does not produce the same effect as with both; an intermediate response is seen.

Section 4.4.2 showed that β- adrenoreceptor stimulation was in part responsible for a cardioprotective effect following a short period of activation. However, it is known from long clinical experience and multiple studies that sustained activity at this receptor family does produce deleterious consequences for the heart at both the subcellular and whole organ level, including mitochondrial dysfunction, hypertrophy, heart failure, and death (Dorn, Tepe et al. 2000, Appukuttan, Kasseckert et al. 2012, Rau, Wang et al. 2015). The potential for these untoward consequences of chronic receptor stimulation, as well as the unintended off-site consequences in the whole organism of receptor activation, mean that it is important that this work confirms prior findings of a receptor independent pathway to cAMP linked cardioprotection.

In order to explain why *both* PKA and Epac must be stimulated in order to observe this effect, the most obvious explanation may be that they have differing downstream effectors of this cardioprotection and so exhibit synergism.

Activity of PKA following stimulation with isoprenaline is known to be associated with glycogen depletion (Khaliulin, Parker et al. 2010); this secondarily leads to elevations of lactate as well as hydrogen ions, leading to increases also in sodium and calcium levels (Cross, Opie et al. 1996). Further others have described this depletion of glycogen, by these mechanisms or unknown others, to disassociation of mitochondrial hexokinase II. This enzyme appears to be an integral part of the MPTP apparatus (Pasdois, Parker et al. 2012) and so loss of this component leads to inhibition of the MPTP. Thus by this means PKA activity leads to cardioprotection;

however, other authors have linked alternative signalling pathways through PKA to cardioprotection; significantly phosphorylation of GSK-3β (Juhaszova, Zorov et al. 2004, Gomez, Paillard et al. 2008), but also others less directly connected to the MPTP such as IKK/IκB and phosphodiesterases (Omori and Kotera 2007, Zhang, Wang et al. 2013).

The reasons and mechanisms by which Epac may have a cardioprotective effect, or may potentiate the effect due to the activity of PKA are less clear. Others have not found that CPT stimulation in similar models produces an isolated protective effect (Duquesnes, Derangeon et al. 2010, Khaliulin, Bond et al. 2017). It has been speculated that activation of PKCε, shown to be necessary for cardioprotection in Section 4.4.2 and known to be activated in Temperature Preconditioning as the archetype for this phenomenon, is a downstream consequence of Epac activity.

4.5.4.3 The immature perfused heart shows increased resistance to injury

These results in the immature heart also show the increased resistance of the developing heart to ischaemia & reperfusion injury. The developing heart does not display the same injury as that of the adult from a time- matched ischaemia/reperfusion injury; the duration of ischaemia needed to be extended from 30 to 50 minutes to obtain an injury which was comparable to that shown by the adult heart after a 30 minute ischaemic stimulus. This is consistent with previous work showing a significant resistance to injury at this developmental stage, with an increase towards adulthood.

4.5.4.4 Immature heart is protected against injury by a combination of PKA & Epac stimulation

Once the injury was significant enough that a hypothetical cardioprotective effect could be observed, a significant reduction in infarct size and in biochemical markers of injury was seen with 8-Br perfusion. This effect was only convincingly seen with 8-Br; 6-Bnz did seem to produce a reduction in CK activity relative to control, but this

effect was not matched with the change in infarct size; whilst CPT seemed to have no significant effect.

Thus, it seems as though in the immature heart activity of both PKA and EPAC is necessary for a protective effect just as in the adult. PKA activity alone has a small protective effect, and EPAC activity alone does not seem to produce protection. Only by simultaneous activation does the maximal protective effect become apparent, implying a synergistic mechanism of action.

4.6 Summary

This chapter has examined physiological and pharmacological differences between the immature and adult whole perfused heart. It has been shown that the physiological responses to signalling at the β- adrenergic receptor are similar between the two age groups.

However, moving to the pathological situation of simulated ischaemia and reperfusion injury, the experiments described here show that the immature heart is indeed more resistant to injury than the adult. Whilst previously described cardioprotective interventions were shown to be ineffective in the immature heart, this may be because of the intrinsically elevated resistance to injury either acting via the same mechanism as the putative cardioprotective intervention or because the injury becomes too small to detect a benefit.

Further, in the adult heart it was shown that inducing activity downstream from the β-AR was also sufficient to produce cardioprotection, although this required simultaneous activation of PKA and EPAC; either on their own was not adequate. A temporally equivalent injury in the 14-day old heart did not show this effect; however when the duration of ischaemic injury was extended to 50 minutes from 30 minutes to produce a histologically equivalent injury the same cardioprotective effect was seen.

A key question that arises from these observations then is what makes the immature heart have an intrinsic protection against I/R injury. Further, since the adult heart shows a cardioprotective effect resulting from activity throughout the β receptor signalling axis, does that cardioprotective effect stem from differences in expression through that pathway.

Chapter 4 examines the proteome of the immature and adult heart and attempts to explain these observations with reference to the abundance of some of these key intermediaries. Chapters 5 and 6, examining isolated cardiomyocytes and mitochondria respectively, examine the differences at the cellular and organelle level in the immature heart and in their response to these same agonists.

5 The effect of cAMP analogues on freshly isolated quiescent cardiomyocytes exposed to simulated I/R in suspension

5.1 Introduction

In Chapter 3 it was shown that transient activation of β-AR receptor of the whole perfused heart is cardioprotective. The cardioprotection is also seen when directly activating the cAMP/PKA/Epac signalling pathways without directly activating the β-AR receptor in both adult and immature heart. This was done using cAMP analogues which are permeable and can directly activate PKA or Epac or both. Further, one of the underlying hypotheses of this thesis is that protection from I/R injury is due to inducing desensitisation of the MPTP in cardiomyocytes in response to elevated Ca^{2+}. However, in addition to cardiomyocytes, the cardiac tissues also contain other structures including the vasculature which can also influence vulnerability to I/R. It is important therefore to show that the protection from injury results from an intracellular process within the cardiomyocyte, rather than any other cell type in the heart.

5.1.1 The Use of Cardiomyocytes as an Experimental Model

To establish whether the cardioprotective effects of the cAMP analogues in whole hearts are due to direct action on the cardiomyocytes and to other cell types, it is then necessary to study their cardioprotective effects using isolated cardiomyocytes. Although Langendorff perfused heart has been used for over 100 years, success at isolating cardiomyocytes using enzymatic digestion was only achieved in the late 1970s. Whilst there are advantages and disadvantages to using the *ex vivo* perfused heart as an experimental model, the same can be said of the isolated cardiomyocytes. Table 5-1 lists key advantages and disadvantages of both models.

Method	Advantage	Disadvantage
Langendorff Perfused Heart	• Technically Straightforward • Physiologically relevant measures of function • Allows assessment of gross functional and electrophysiological measurements. • Good for histological and molecular studies	• Loss of neurohumoral regulation • Absence of physiological response to preload & afterload • Time limited preparation- function changes with time • Mostly uses Non-physiological metabolic substrates (e.g. glucose) • Measurement of function with LV balloon is invasive (esp. in immature) • Endpoints cannot be extrapolated to cell levels due to heterogeneity (SAN, atrial, ventricular cells and other cell types)
Freshly Isolated Cardiomyocyte	• Allows measurement of intracellular changes not accessible in whole heart (e.g. channel activities & E-C coupling) • Direct assessment of effects on individual cells • Allows culture of cardiomyocytes for less temporally limited experimentation	• Significant injury incurred in isolation process • Selection bias- only stronger cells survive isolation • Can be technically challenging with high failure & spontaneous death rate • They are unloaded • Ca^{2+} tolerant • Quiescent • Characteristics change in culture

Table 5-1 Comparative advantages and disadvantages of perfused heart and isolated cardiomyocyte as a model of ischaemia & reperfusion injury.

Freshly isolated cardiomyocytes are the gold standard for electrophysiological studies including patch clamping and whole cell studies. Although isolated cardiomyocytes are sometimes maintained for several days in culture, it has been shown that such cells do show morphological and electrophysiological changes (see Mitcheson (Mitcheson, Hancox et al. 1998). Freshly isolated neonatal myocytes can be cultured are good for investigating characteristics of differentiating neonatal cells.

Freshly isolated myocytes can also be cultured and kept for more than one day but they do undergo electrophysiological and morphological changes.

Considering cell culture as a means of studying the cardiomyocyte, there are some advantages. Clonal expansion of the population allows a relatively homogenous group of cells to study. Further, the separation of an experiment from the process of obtaining the cardiomyocyte allows for a recovery from damage sustained during isolation (Borg and Terracio 1990). Cells in culture may also be studied over the longer time scales than are possible just examining the freshly isolated cardiomyocyte for instance for genetic, hormonal, or environmental manipulation. Also, for repeated observation, a cell should be stationary and the location marked. This is much more straightforward in a cell culture setup (Diaz and Wilson 2006). However, these advantages are not pertinent to the questions posited here. There are significant disadvantages, also. Marked morphological and ultrastructural changes have been noted in cardiomyocytes in cell culture (Decker, Simpson et al. 1990, Decker, Behnke-Barclay et al. 1991) which may markedly alter their electrical and contractile properties. They therefore cannot be used for electrophysiological studies, nor for assessment of contraction.

These issues are avoided through the use of freshly isolated cardiomyocytes, which allow for real time visualisation of at least some aspects of excitation contraction coupling, and ready changes to the extracellular environ in order to alter the ionic gradients or add pharmacological stimulation. Indeed in part due to these advantages, isolated cardiomyocytes have been used to study cardiac conditioning for some years, with a key early example using isolated cardiomyocytes to model ischaemic preconditioning (Vander Heide, Rim et al. 1990), whilst the enzymatic separation of cardiomyocytes from the heart has been viable for even longer (Altschuld, Gibb et al. 1980, Altschuld, Hostetler et al. 1981).

However, the use of this model does increase the degree of abstraction from the clinical phenomenon of ischaemia/ reperfusion injury. A key feature of this for instance is hypercontracture, in which disruption of the cytoskeleton and sarcolemma occurs leading to a marked release of Ca^{2+} into the affected cells (Piper, Garcia-Dorado et al. 1998, Piper, Meuter et al. 2003). Vascular effects, such as

alterations in vessel permeability as an inflammatory consequence, cannot be observed in this model. The various whole heart preparations are more appropriate models of these effects. The isolated cardiomyocyte, however, offers a suitable vehicle for the examination of models of ischaemia & reperfusion and potentially protective interventions.

5.1.2 Modelling Reperfusion injury

Numerous models are available to simulate ischaemia & reperfusion injury. An obvious possibility for modelling would be to simply immerse the isolated cardiomyocytes in an oxygen-deplete medium lacking in metabolic substrates. However, isolated cardiomyocytes are inherently quiescent, and lacking in the load to which they would be exposed working as part of a heart. Therefore their metabolic requirements, and so rate of injury in these conditions, are very low. A widely used alternative is to bypass the ischaemic phase due to these difficulties and replicate the conditions in the medium that occur during reperfusion; that is of high oxidative stress and high Ca^{2+} concentrations. The production of reactive oxygen species is a key feature of early reperfusion injury (Garlick, Davies et al. 1987, Grill, Zweier et al. 1992, Sun, Wang et al. 2005); and they play a significant role in the deleterious effects of reperfusion (Josephson, Silverman et al. 1991).

H_2O_2 is a key member of the family of ROS produced during reperfusion. The consequences of its actions include direct damage to organelles and DNA, but also oxidation of ion channels and transporters and interruption of signalling cascades through activation of intermediaries such as CaMKII; all of which can lead to Ca^{2+} overload in the intracellular environment of cardiomyocytes (Gen, Tani et al. 2001, Zima and Blatter 2006, Erickson, He et al. 2011). It can also lead to stimulation of the RyR Ca^{2+} release mechanism in SR, as well as increase NCX activity, all of which further contribute to Ca^{2+} overload in the cardiomyocyte (Boraso and Williams 1994, Goldhaber 1996, Belevych, Terentyev et al. 2009), an important intermediate step leading to cell death (Garcia-Dorado, Ruiz-Meana et al. 2012). The use of a buffer enriched in H_2O_2 with a high concentration of Ca^{2+} recreates these conditions. This laboratory has previously tested H_2O_2 at 200 µM and found that it can produce

hypercontraction in (contracting) cardiomyocytes relatively rapidly. It therefore seems reasonable to attempt to use it as a source of injury in quiescent isolated cardiomyocytes.

5.2 Aims

The aim of this chapter was to establish whether protection by cAMP/PKA/Epac signalling seen in the whole heart can also be seen in isolated cardiomyocytes, and to show that that protection is similar to that elicited by direct MPTP inhibition.

1) Optimise conditions for monitoring the viability and morphology of isolated adult & immature cardiomyocytes in suspension
2) Show that exposure to H_2O_2 and a high concentration of Ca^{2+}, similar to intracellular conditions on reperfusion, triggers cardiomyocyte death in this model
3) Examine the effects of cAMP analogues incubated with the cardiomyocytes on this simulated reperfusion injury
4) Compare that with the protective effect of CsA to demonstrate interaction with the MPTP as a mechanism for putative cardioprotection

5.3 Methods

5.3.1 Cardiomyocyte isolation

The methods by which cardiomyocytes were isolated is discussed in detail in Chapter 2, including the composition of the various solutions used. Briefly, P14 and P28 as well as adult rats were stunned by concussion and killed by cervical dislocation. Their hearts were rapidly extracted and immersed in chilled buffer B.

The apparatus for the isolation of cardiomyocytes is shown in Figure 5-1. The hearts were cannulated via the aorta and perfused with solution B for at least 2 minutes. The perfusate flow rates were constant, at 2.8 ml/min for P14 hearts, 6.4 ml/min for P28, and 8.6 ml/min for adults based upon previous optimisation work for this technique in our laboratory. Once stable, perfusion was switched to the EGTA containing solution C in order to chelate Ca^{2+} for a further 4 minutes, then perfusion was switched to solution D containing a collagenase and protease which perform the digestion. As this technique for cardiomyocyte isolation begins with the same *ex vivo* perfusion process used in Chapter 4, the exclusion criteria for recognising failed or problematic isolation is identical to that discussed in Section 4.3.2. Once a heart had been successfully digested it was included in the analysis.

The end point for digestion was judged subjectively by touch but was relatively consistent within an age group. Ca^{2+} was then slowly reintroduced with solution E whilst the heart was removed from the cannula and gently pulled apart with forceps. This solution was agitated at 37 °C for 4 minutes, then filtered and allowed to sediment. The supernatant was then removed, replaced by solution F then G for 5 minutes each respectively in order to slowly increase the Ca^{2+} concentration back to physiological. A droplet of the resulting suspension was then examined under a 20x light microscope to confirm the success of the isolation. The isolation was regarded as having failed if more than 30% of the observed cells across multiple fields had lost their characteristic shape (Figure 5-2). These cardiomyocytes were then used for both the experiments involving the effect of cAMP analogues and CsA on simulated

reperfusion injury in Sections 5.4 and 5.4.3, and also the effect of cAMP analogues on contractility and transients in a model of ischaemic injury in superfused cardiomyocytes, described in the following chapter.

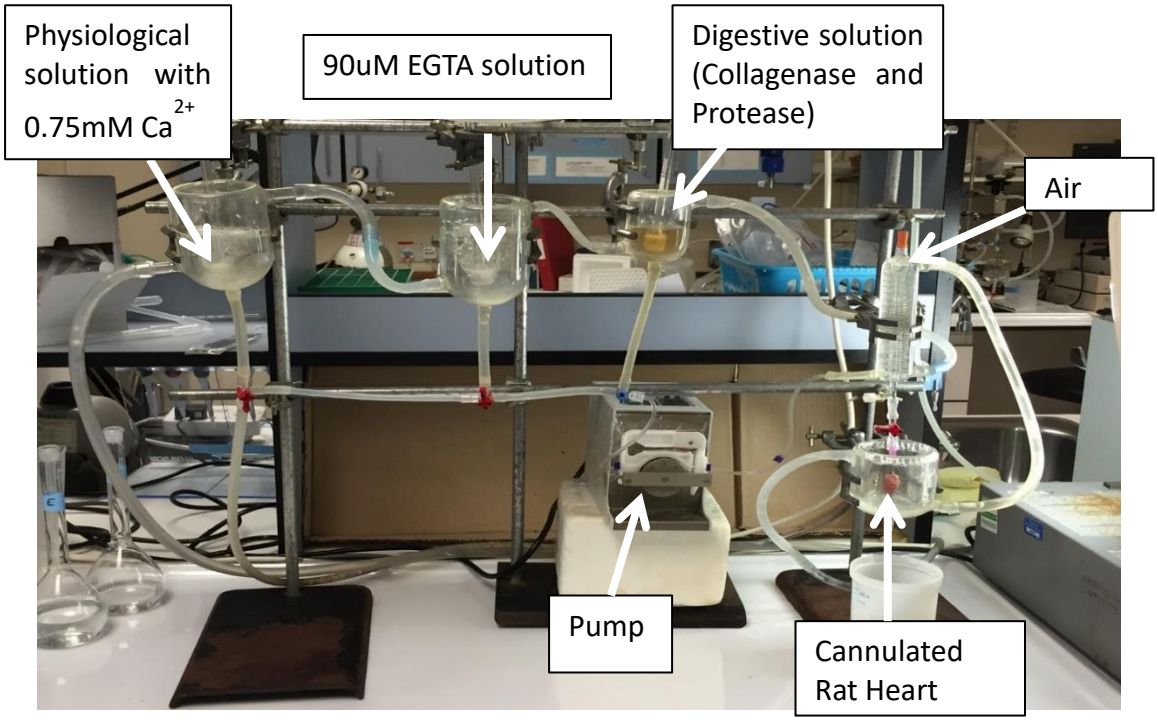


Figure 5-1 Perfusion apparatus for cardiomyocyte isolation experiments.

5.3.2 Experimental protocols for Incubation Experiments

5.3.2.1 cAMP analogue experiments

For these experiments, the cardiomyocyte isolate was divided into 5 conical flasks, each of which would be used for the incubation of a different experimental condition. These experiments were performed in an immature P14 group (n=5 per condition), and in an adult group of isolated cardiomyocytes (n=6 per condition). It is important to be clear about sample sizes with respect to these experimental conditions- because a digested heart provided a very large number of cardiomyocytes, and to exclude unaccounted variation in the isolation process, cardiomyocytes from a particular heart were used to test all experimental conditions together. However, in the analysis by microscopy, 5 random fields from each condition for each heart were analysed. The total numbers of hearts used are shown accordingly in Table 5-2.

Age Group	*Numbers of hearts digested*
Adult	6
P14	5

Table 5-2 Numbers of hearts from each age group tested digested and used in experiments incubating cardiomyocytes with cAMP analogues, divided by age group. Each digested heart was used for all experimental arms- control, simulated I/R and simulated I/R + drug; in order to limit variation.

Control- This aliquot was agitated and warmed to 37 °C in a water bath, but had no further intervention. It served as a baseline of the intrinsic death rate of the population of cells after isolation

Simulated Ischaemia/Reperfusion (I/R): as for control but with the addition of 0.5 mM H_2O_2 and 3 mM $CaCl_2$ and the glucose omitted from the buffer.

Simulated I/R + drugs: as above but with the addition of a cAMP analogue, incubated in the suspension for 10 minutes. The supernatant containing these drugs was then removed prior to the addition of the H_2O_2 and $CaCl_2$ in an equivalent volume of unadulterated buffer. Concentrations used were 5 µM 8-Br, or 10 µM 6-Bnz, or 10 µM CPT.

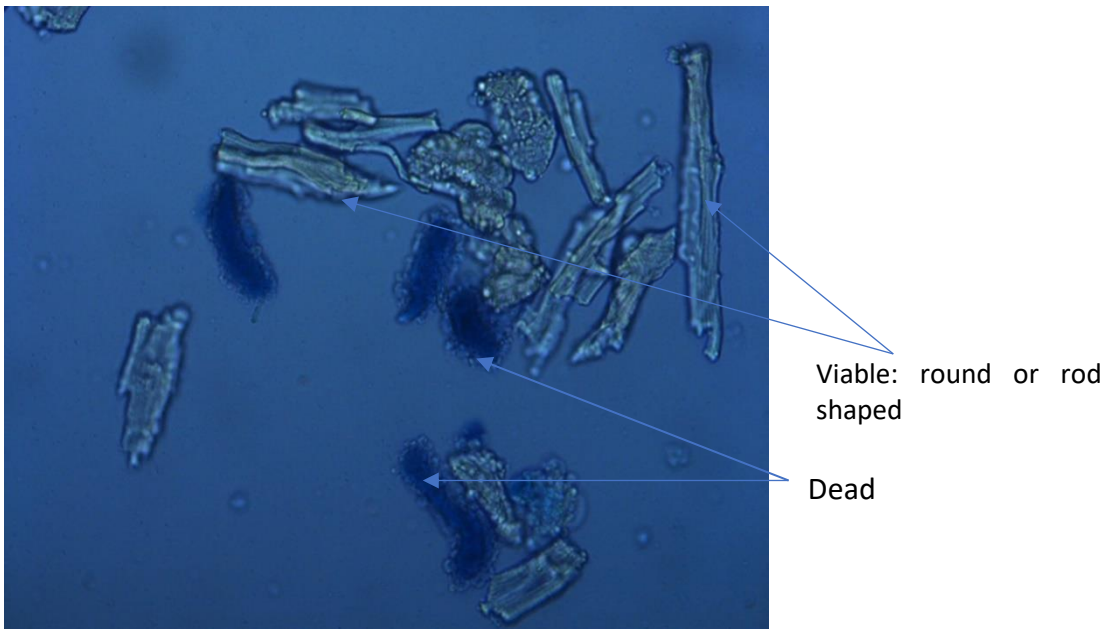


Figure 5-2 Example of appearances of isolated cardiomyocytes.

Those cells which remain viable are those which retain their characteristic rod shape; those that are significantly injured become rounded, and once their membrane integrity is compromised take up Trypan Blue.

5.3.2.2 CsA experiments

The cardiomyocyte suspension was divided into 3 aliquots of 5 ml, with each for a separate experimental condition. These experiments were performed in a P14 group (n=5 per condition), an adult group (n=5 per condition) and an additional P28 group (n=6 per condition)

Age Group	*Numbers of hearts digested*
Adult	5
P28	6
P14	5

Table 5-3 Numbers of hearts from each age group tested digested and used in experiments incubating cardiomyocytes with CsA, divided by age group. Each digested heart was used for all experimental arms- control, simulated reperfusion and simulated reperfusion + CsA; in order to limit variation.

Control- this aliquot was gently shaken and incubated at 37 °C for 120 minutes but was left otherwise untouched.

Simulated Reperfusion- as for control but with the addition of 0.5 mM H_2O_2 and 3 mM $CaCl_2$.

Simulated I/R + CsA- as above but with the addition of 2 µM CsA, incubated in the suspension for 10 minutes prior to simulated I/R.

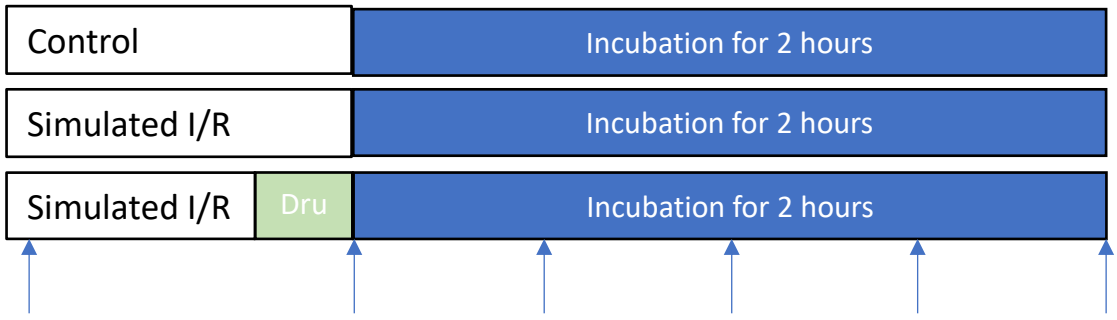


Figure 5-3 Experimental protocols for investigating the effects of I/R with or without drugs on viability and morphology of cardiomyocytes isolated from adult and 14-day hearts. Arrows indicate time of assessing viability and morphology of cardiomyocytes. Time= 0 for results data to follow refers to start of incubation phase.

For both of these sets of experiments, samples of the suspensions were then taken initially and at intervals of 30 minutes until 120 minutes. Drops of each suspension

were placed on a cover slip with the addition of 5.5µl of Trypan Blue (0.4%, Sigma) and examined under 20x optical magnification. 5 fields were randomly selected and images captured.

The images were assessed using the software ImageJ (NIH, Bethesda, US) which was used to count dead, hypercontracted, and rounded cells. A mean for each time point across each sample and experimental condition was calculated, and then normalised to the viability from the initial sample which was defined as 100% to allow cross experimental comparison.

Statistical analysis where noted was by way of two- way repeated measures ANOVA using the Bonferroni correction. This was performed in SPSS v. 23.0 (IBM, New York, US). Data are presented graphically as means ± SEM.

5.4 Results

5.4.1 Viability of Cardiomyocytes following Isolation Protocol

After the process of isolation, the cardiomyocyte suspension was observed and the proportionate viability estimated. This varied significantly between groups but was relatively consistent within each age group. The mean proportionate viability immediately after isolation is shown for each age group in Table 5-4. Values are calculated from all isolations in a given age group, as these are not particular to any experimental protocol, and are expressed as a proportion of viable to non-viable and dead across the fields observed.

Age Group	*Relative Mean viability ± SEM*
P14	57.6± 2.9%
P28	59.1 ± 5.6%
Adult	70.3 ± 3.8%

Table 5-4 Mean proportion of viable cardiomyocytes obtained following isolation for each age group studied ± SEM. Total numbers of hearts for each group- P14= 10, P28= 6, Adult= 11.

5.4.2 The effect of cAMP analogues on adult and immature cardiomyocytes exposed to simulated I/R

Figure 5-4 and Figure 5-5 show the results of pre-incubation of the cAMP analogues on adult and immature cardiomyocytes prior to simulated reperfusion injury. For the adult group, after 120 minutes observation, the viability of the control group had fallen to 78 ± 1.73% of the initial. In this group, exposure to the simulated reperfusion condition with H_2O_2 and Ca^{2+} without any additional intervention caused that viability to fall to 46.3 ± 5.90%. The cAMP analogues caused a stepwise improvement in that

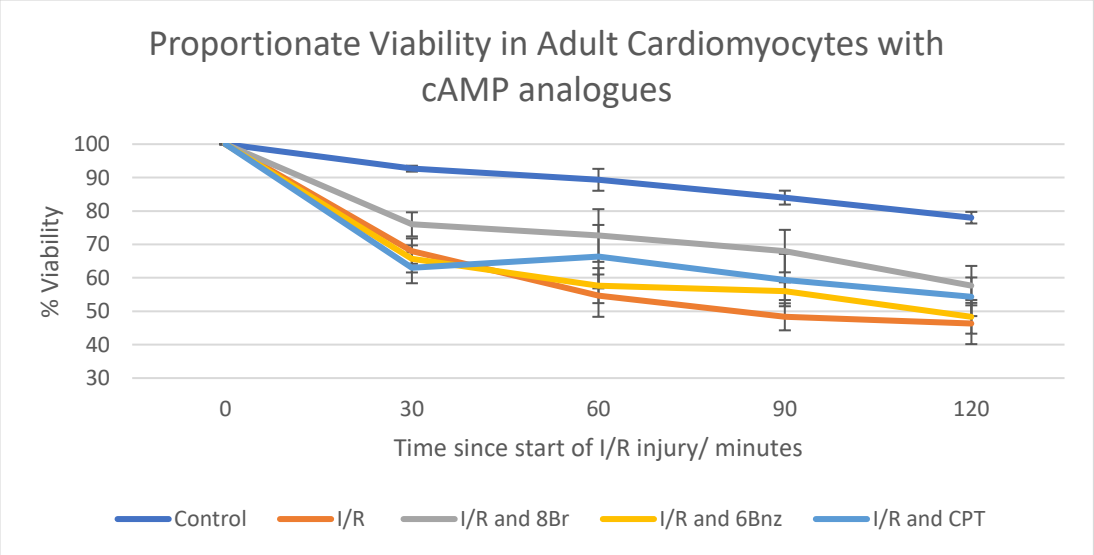


Figure 5-4 Change in proportionate vulnerability of adult (n= 6 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with cAMP analogue. Normalised to starting viability defined as 100%. Points plotted as mean ± SE.

viability by the end of each experiment. CPT and 6-Bnz caused a non-significant improvement in viability but 8-Br was significantly improved vs. the simulated reperfusion condition alone ($p = 0.02$).

In the P14 group, a similar pattern was observed. A striking finding was the increased death rate in the control group- after 120 minutes the viability was 55 ± 3.46%, compared to the adult group which was 78 ± 1.73%. The same pattern of changes with the different experimental conditions was seen, however, notwithstanding the increased background rate of death; non-significant improvements in vulnerability with 6-Bnz and CPT but a significant partial rescue with 8-Br (at 120 min, 35.4 ± 4.98 vs 55 ± 3.46% p=0.0014).

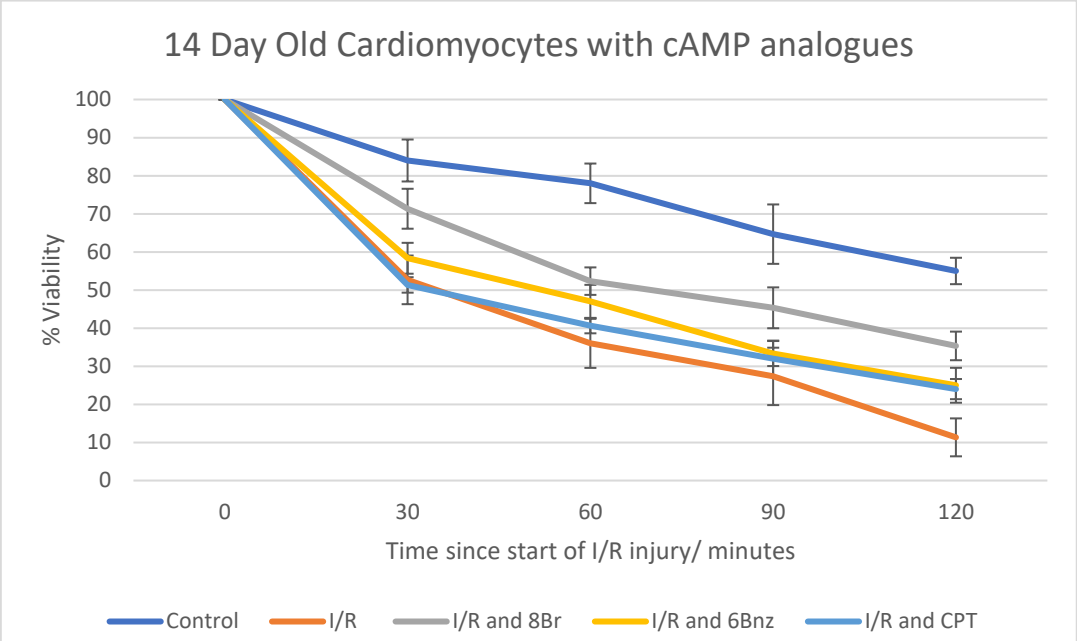


Figure 5-5 Change in proportionate vulnerability of P14 (n= 5 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with cAMP analogue. Normalised to starting viability defined as 100%. Points plotted as mean ± SE.

	Adult/% hr^{-1}	*P14/% hr^{-1}*
Control	14.6 ± 0.9	32.0 ± 5.5
I/R	64.0 ± 3.6*	94.8 ± 5.2*
I/R and 8Br	48.0 ± 3.8	57.2 ± 6.4
I/R and 6Bnz	68.6 ± 4.1*	83.4 ± 4.0*
I/R and CPT	74.2 ± 4.6*	97.2 ± 2.1*

Table 5-5 Initial death rates at 30 minutes for cardiomyocytes incubated with cAMP analogues in simulated I/R conditions expressed as percentage per hour ± SEM. Asterisk= p<0.05 vs same aged control result. Tested by Student's T-test.

5.4.3 The effect of Cyclosporin A on adult and immature quiescent cardiomyocytes exposed to simulated reperfusion injury

The adult cardiomyocytes in suspension showed a change in viability with time as shown in Figure 5-6. After 120 minutes following the start of measurement, the control cells (those with no additional intervention) showed a mean viability of 76±6.5%. This was identical (76±7.4%) to those cells exposed to the simulated reperfusion injury condition, with a high calcium concentration and hydrogen peroxide as well as CsA; those exposed to the injury condition alone showed a significantly lower viability, with a mean of 53±5.3% after 120 minutes.

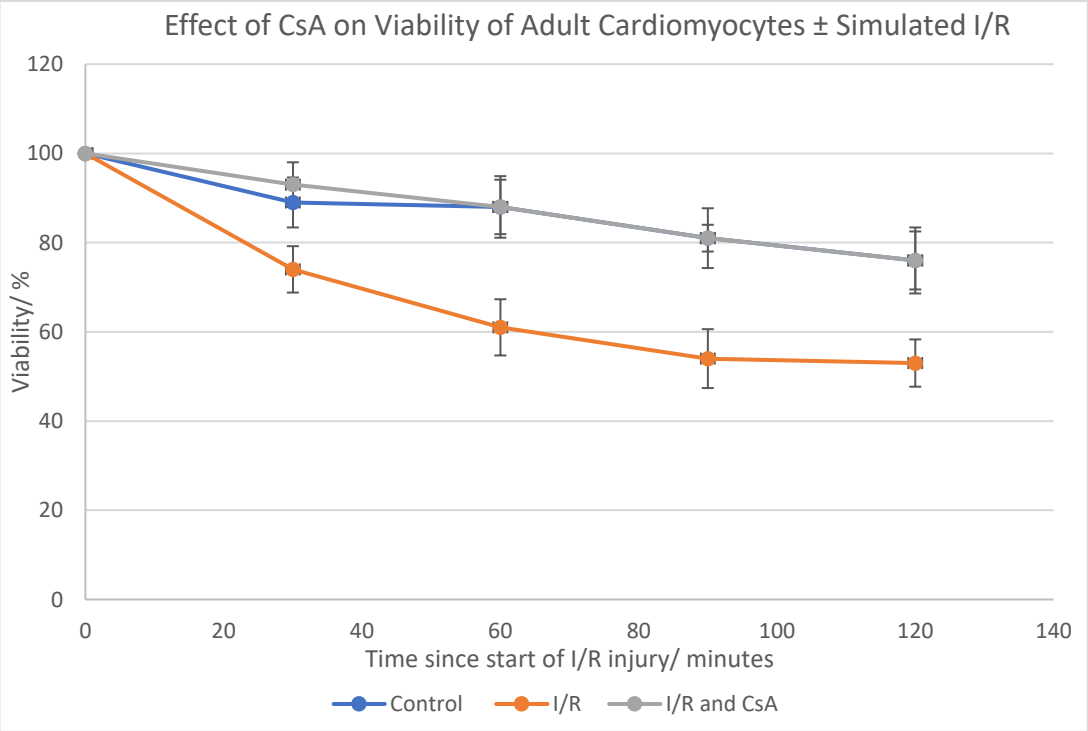


Figure 5-6. Change in proportionate vulnerability of adult (n= 5 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with CsA. Normalised to starting viability defined as 100%. Points plotted as mean ± SE.

This pattern was not repeated in the two immature cardiomyocyte groups, of 28 and of 14 days post- natal. Figure 5-7 shows the pattern of viability vs time in 28 day old hearts, and Figure 5-8 that in 14- day old cardiomyocytes.

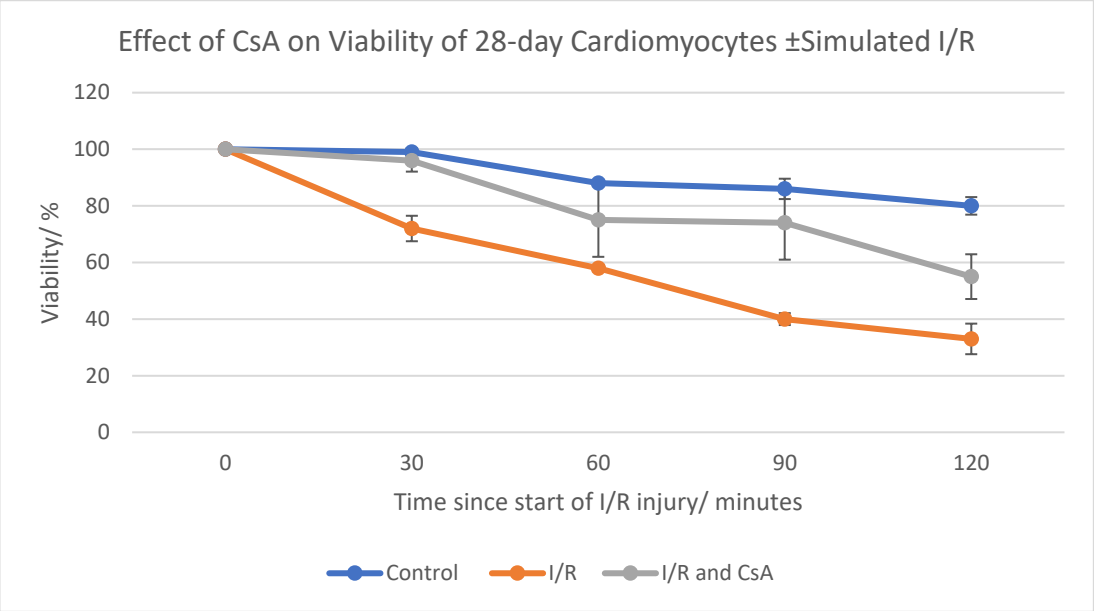


Figure 5-7. Change in proportionate vulnerability of P28 (n= 6 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with CsA. Normalised to starting viability defined as 100%. Points plotted as mean ± SE.

In the 28- day old cardiomyocytes, the viability of cells treated with CsA at the end of 120 minutes incubation was intermediate between those not exposed to a simulated reperfusion injury and those just exposed to the injury with no CsA- that is, there was a partial amelioration of the injury. The control arm in this age- group showed an 80±3.1% viability at the end of the experiment, as compared (not significantly different) to the CsA + reperfusion injury arm which showed a viability of 55±7.9%. However, this was nonetheless significantly greater than the reperfusion injury alone arm, with a viability of 33±5.4% at the end of the experiment.

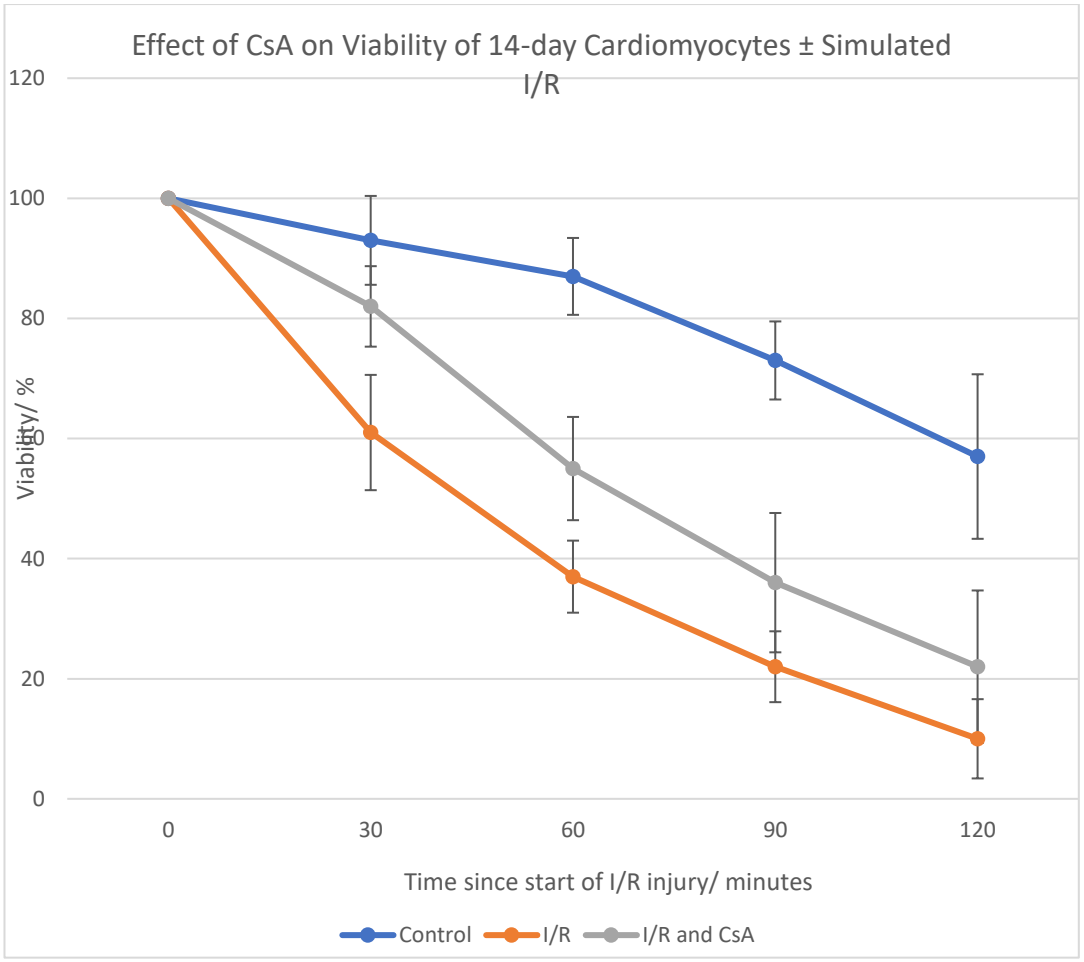


Figure 5-8. Change in proportionate vulnerability of P14 (n= 5 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with CsA. Normalised to starting viability defined as 100%. Points plotted as mean ± SE.

The 14- day old cardiomyocytes did not show any significant difference between the arms containing hydrogen peroxide/ high calcium (the simulated reperfusion injury) with or without the addition of CsA. The control arm in this group showed a greater rate of decline than in either of the two previous age groups, declining to a mean viability of 57% by the end of the two hour experiment; this is approximately 40% greater decline than in the adult age group.

	Adult/ % hr^{-1}	*P28/ % hr^{-1}*	*P14/ % hr^{-1}*
Control	22 ± 5.6	2 ± 1.3	14 ± 7.4
I/R	52 ± 5.2 *	56 ± 4.5 *	78 ± 9.6 *
I/R and CsA	14 ± 5.0	8 ± 3.9	36 ± 6.7

Table 5-6 Initial death rates at 30 minutes for cardiomyocytes incubated with CsA expressed as percentage per hour ± SEM. Asterisk= p<0.05 vs same aged control result. Significance tested by Student's T-test.

An additional experimental outcome for each of these arms was also considered- the cardiomyocyte morphology at each time point, classified into normal i.e. rod-shaped morphology, rounded but not taking up Trypan blue dye, or rounded and blue in colour in response to the stain. The results from this measurement are shown in Figure 5-9 and Figure 5-10.

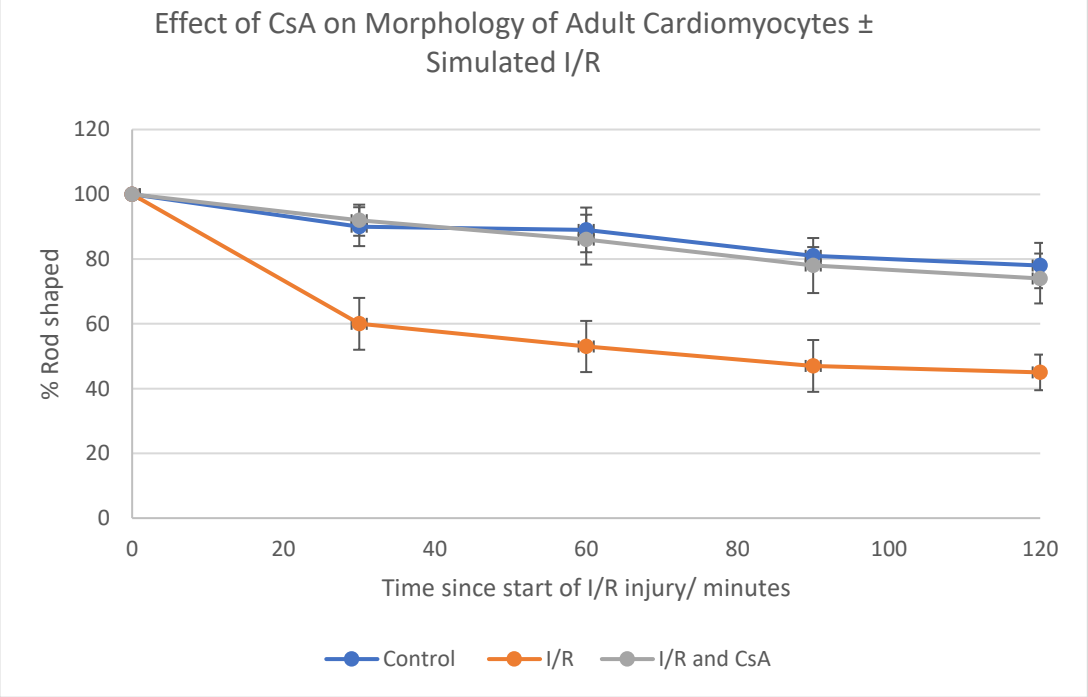


Figure 5-9. Change in morphology expressed as proportion of physiological rod shape of adult (n= 5 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with CsA. Normalised to starting morphology defined as 100%. Points plotted as mean ± SE.

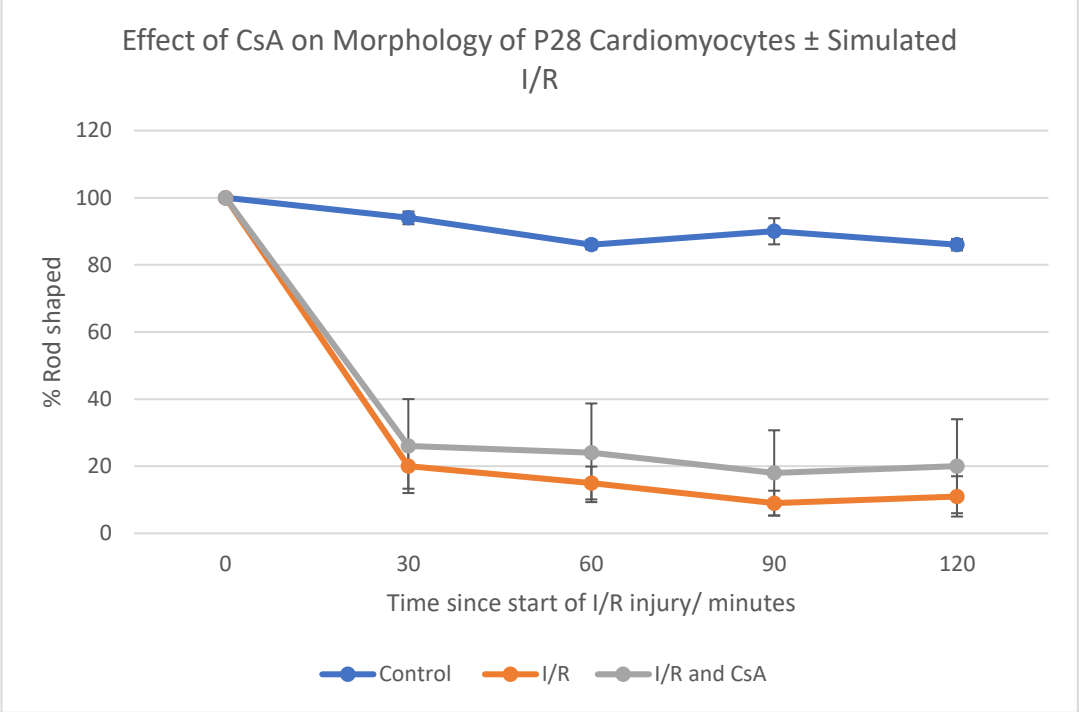


Figure 5-10. Change in morphology expressed as proportion of physiological rod shape of P28 (n= 6 hearts) isolated cardiomyocytes incubated in simulated I/R conditions vs time after incubation with CsA. Normalised to starting morphology defined as 100%. Points plotted as mean ± SE.

For the adult group, the trend in morphology mirrors closely that for viability. At the end of the two- hour experimental period, the proportion of normal rod- shaped cardiomyocytes in the control and CsA/reperfusion group was indistinguishable, at 78±7.0% vs 74±7.7%. However, the reperfusion group showed a marked change in morphology, with the rods making up only 45±5.5% of the identified cells after 120 minutes. This was significantly different from the CsA/reperfusion and control groups ($p<0.01$).

The 28- day old group did not display the same pattern as the adult. In this group, the addition of cyclosporin A to the buffer did not cause surviving cardiomyocytes to retain their rod shape; the CsA/reperfusion and reperfusion arms were indistinguishable. Both of these groups were significantly different to the control arm at every time point measured from 30 minutes. The P14 day group is not shown. Reliably assessing morphology in the immature cells is difficult; the P14

cardiomyocytes are stellate in shape intrinsically, and so differentiating their normal morphology from hypercontracture is challenging using this method. This interaction between developmental changes in morphology and pathological observations makes using this outcome measure in order to assess cardioprotective interventions across age groups less useful.

5.5 Discussion

5.5.1 Summary of findings

In these experiments on viability of cardiomyocytes in suspension, there were three key observations. Firstly, that freshly isolated cardiomyocytes are fragile in suspension, and that the intrinsic rate of death following isolation is higher in P14 and indeed P28 cardiomyocytes than in the adult. Secondly, activation of both PKA and EPAC via incubation with 8-Br prior to simulated I/R injury is ameliorates cardiomyocyte death in both adult and P14 cells. Finally, whilst adult cardiomyocytes show rescue from simulated I/R injury with incubation with Cyclosporin A, P14 cardiomyocytes do not appear to, whilst P28 as an intermediate group show a partial response.

5.5.2 Adult cardiomyocytes in suspension are less vulnerable to death than P14 cardiomyocytes

This work shows that freshly isolated cardiomyocytes from adult and 14-day old hearts, are vulnerable to a simulated reperfusion injury caused by an environment supplemented with H_2O_2 & high Ca^{2+}. This was observed in both sets of experiments involving CsA and cAMP analogues.

However, the magnitude of this injury seemed to be greater in the 14- day old group than in the adult; after 120 minutes the viability had been reduced to 53% in the adult vs. 10% in the immature group (CsA group of experiments; in the cAMP analogue group, carried out separately, the pattern is the same although the numerical values differ at 78% for adult and 55% for P14).

These observations are in contrast to the prior observation (Riva and Hearse 1993, Ostadalova, Ostadal et al. 1998) that there is a bell- shaped vulnerability curve to the response to I/R; it is thought that vulnerability to injury falls until a minimum at 14-days of age, before increasing again towards adulthood. However, these observations were made on the whole heart, rather than isolated cardiomyocytes. My previous findings, outlined in Chapter 3, included experiments on whole heart perfusion of adult and 14- day old hearts, confirmed the finding that immature hearts

are less vulnerable than adult hearts to damage from reperfusion injury. Therefore, these findings are not representative of findings in the literature or my own prior data on whole hearts. This may reflect damage to cardiomyocytes as they are isolated. The process by which cardiomyocytes are isolated involves perfusion for variable periods with collagenase and protease; these enzymes by nature are damaging. Further, the endpoint for digestion of hearts was not constant either within or between age groups; for each heart, it was determined subjectively. This may have led to a systematic overdigestion of the immature hearts leading to this increase in injury, seen both in the control arm and in the experimental groups deliberately exposed to intervention.

Another distinction between this present study, and those prior, is that this study attempted to isolate the effect of simulated reperfusion injury distinct from that caused by ischaemia; this is uniquely possible in the isolated cardiomyocyte preparation. In the previously studied whole heart preparations, the hearts were exposed to the combination of ischaemia and reperfusion; the addition of ischaemia may possibly account for the difference, or a deficiency in the representation of reperfusion injury in this method of simulating it.

There are however, alternate explanations. There is a strong selection bias in the method of digestion; if there is a spectrum of vulnerability in cardiomyocytes, those most resistant to injury will survive resulting in a systematic bias in results. The environment in which isolated cardiomyocytes sit, whilst the buffer is carefully composed to attempt to recapitulate the usual extracellular milieu, is not the same as their physiological condition in the heart (Nippert, Schreckenberg et al. 2017). One clear difference is that in the heart, cardiomyocytes sit in an organised structure; communicating between themselves electrically and chemically through gap junctions, paracrine signalling and other means (Vornanen 1996). In suspension, these cardiomyocytes are mechanically unloaded through this lack of structure, which is clearly an aphysiological situation.

Protective mechanisms may exist that depend upon these modalities of intercellular communication; these would be disrupted in this isolated cardiomyocyte preparation, and so might explain this observation; these observations may well be

different in a cardiomyocyte culture in media. Also, cardiomyocytes also are not the only cells present in an intact heart, nor is contractile tissue the only tissue type present. It is possible that, for instance, cardiac fibroblasts, or the cardiac vasculature has an important role to play in protection from injury; these effects would necessarily not be apparent in a suspension of isolated cardiomyocytes.

5.5.3 MPTP inhibition by CsA is protective against simulated reperfusion injury in isolated adult but not immature cardiomyocytes

It is thought that a common final pathway for cell death following reperfusion injury involves opening of the mitochondrial permeability transition pore (Ong, Dongworth et al. 2015). The hypothesis for this set of experiments was therefore that administration of a known inhibitor of that pore would be protective for cardiomyocytes of any age exposed to a simulated reperfusion injury with agents thought to act by promoting pore opening. In these experiments, I showed that CsA does completely ameliorate the effect of reperfusion injury on isolated cardiomyocyte viability in cells isolated from adult hearts. However, this effect was only seen to an intermediate extent in 28- day old cardiomyocytes and was completely absent from the 14- day old cardiomyocytes.

It has been previously shown, albeit to a lesser extent, that CsA is capable of inhibiting cell death from reperfusion injury in adult cardiomyocytes (Griffiths, Ocampo et al. 2000). That this agent completely reverses the increase in death from the administration of the injurious stimulus corroborates our model of reperfusion injury as causing damage in the mode that we had hypothesised *a priori.*

It is interesting to note that this finding is not replicated in immature cardiomyocytes. A statistically significant improvement was seen in the 28- day old cells, placing the group treated with CsA in an intermediate position between the control and reperfusion injury arms. In the 14- day old group, there was no protective effect found by administration of CsA. It may be supposed that the MPTP in 14-day old cardiomyocytes is much less prone to opening *per se*, or in any event by the stimuli I

have used to simulate reperfusion injury, and so that the administration of CsA does little to modify the process of injury and cell death in this group.

However, a relative increase in injury and death rate was seen in the immature cells even in the absence of any damaging intervention. Therefore, this observation of a lack of effect must be treated with a little caution, given the observed increase in death rate in immature cells not exposed to any injurious stimulus. With this low viability, a putative effect would be difficult to observe. Nevertheless, even with this increase in spontaneous death rate, there is a significant difference in response in the 14- day old group compared to the adult.

5.5.4 Combined PKA & EPAC stimulation ameliorates death rate from simulated reperfusion injury in both adult & immature isolated cardiomyocytes

These experiments have shown that pre- incubation with 8-Br prior to simulated reperfusion injury can significantly reduce cardiomyocyte death in both adult and immature cardiomyocytes. Either 6-Bnz or CPT alone did not have this effect. It therefore appears as though the effect is dependent upon synergistic activation of both PKA and EPAC, and that neither is adequate to produce a protective effect alone. This is consistent with the effects seen on the whole heart in Chapter 3.

It also therefore seems that the cardioprotective effects of these agents is not dependent upon vascular, or other organ-level pharmacodynamic effects, or indeed upon intracellular communication via gap junctions. The cardiomyocytes in these freshly isolated preparations exist as single cells in suspension and so any effect seen due to these agents is most likely a consequence of intracellular action alone.

It is notable that whilst the cAMP analogue 8-Br provided partial rescue of the P14 cardiomyocytes from simulated reperfusion injury, incubation of CsA with cardiomyocytes of this age does not. Whilst this may be an artefact of the preparation used for the CsA experiments having an unexpectedly high death rate so obscuring benefit from CsA, it may also hint at the regulation of mitochondrial permeability being governed by alternate mechanisms or mediators in the immature heart than CsA.

Chapter 6 next examines some of the intracellular consequences of perfusion with the cAMP agents, and therefore provides some insight into their possible mechanism of action.

6 The effect of cAMP analogues on contractility & Ca^{2+} cycling in isolated adult cardiomyocytes under both physiological and simulated ischaemic conditions

6.1 Introduction

Previously, in Chapter 4, it was demonstrated in the whole heart that analogues of cAMP can produce protection against ischaemia and reperfusion injury, and that that protection depends upon joint stimulation of both PKA and EPAC. In Chapter 5 it was shown that in a model of reperfusion injury those same agents were protective in quiescent freshly isolated cardiomyocytes. However, it is not clear what pharmacodynamic effects these agents are having upon the cardiomyocyte, and if these effects may explain the observed protective properties. Furthermore, it is important to establish cAMP/PKA/Epac signalling pathways involvement in Ca^{2+} cycling and E-C coupling using cAMP analogues. As mentioned in the previous chapter, cardiomyocytes are used to study E-C and change in Ca^{2+} cycling using Ca dyes. However, whereas studies involving I/R using intact perfused hearts is well established, such an intervention does not work in the case of isolated perfused cardiomyocytes. These myocytes are unloaded and when perfusion is stopped, it will take a very long time to see changes leading to death. Additionally, single myocytes do die spontaneously with time. Anoxia has been used (N_2 bubbled buffer) but this also takes a long time. Therefore, more than 20 years ago, the team in Bristol have adopted a chemical hypoxia approach to study insults in cardiac perfused myocytes.

6.1.1 Chemical Hypoxia model

The purpose of these experiments is to assess the changes in contractility that occur under conditions of cardiac ischaemia. The most obvious direct way to study this would be to remove oxygen, and metabolic substrates from the perfusion medium. Chemical hypoxia (addition of Cyanide to solutions perfusing cardiomyocytes) has been widely used and its metabolic effects and effects on Ca and contractility reflect

changes in myocytes exposed to anoxia(Griffiths 1997, Griffiths, Lin et al. 1998, Williams, Kerr et al. 2000, Williams, King et al. 2001, King, McGivan et al. 2003). Cyanide is a complex IV mitochondrial inhibitor (Ball and Cooper 1952) and so inhibits mitochondrial production of ATP via oxidative phosphorylation similar to what occurs during anoxia. Superperfusion of field stimulated cardiomyocytes triggers changes that are similar to is seen during anoxia of cardiomyocytes and also during I/R in intact heart. Like anoxia, metabolic inhibition of field stimulated cardiomyocytes induces a gradual cessation of contractility associated with rigor (since the myocytes are not loaded, if they hypercontracted, they do not relax). Upon reperfusion with normal buffer, the myocytes can either recover contractility or hypercontract further and die (confirmed using trypan blue). More importantly, these changes are paralleled by changes in cytosolic Ca^{2+} that are similar to what is seen in I/R of intact heart (Figure 6-1).

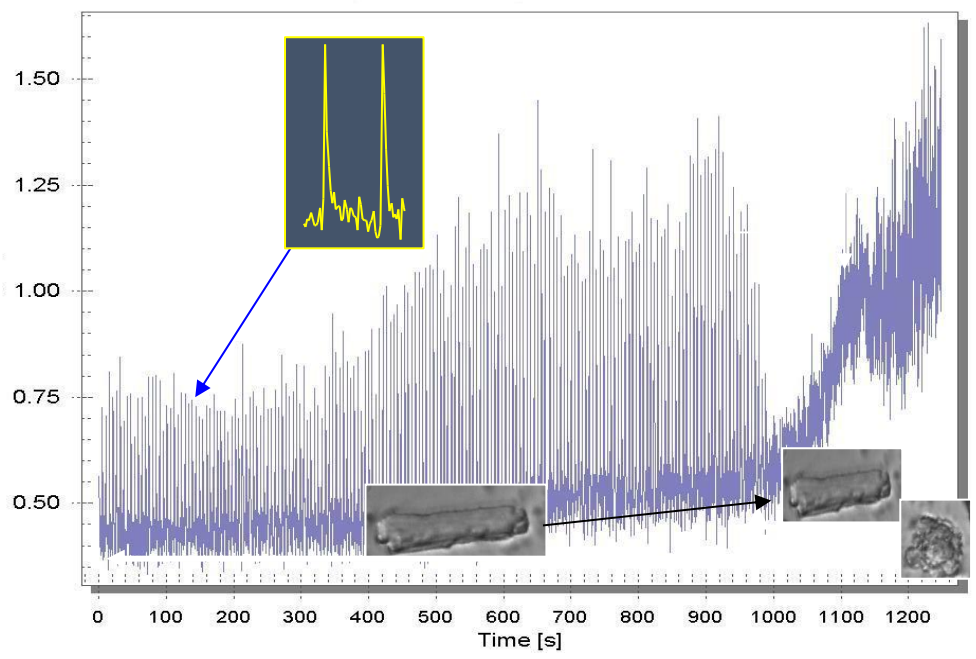


Figure 6-1 Example Ca^{2+} transient trace. Isolated perfused adult rat cardiomyocyte and stimulated at 0.2Hz to measure Ca2+ transients and monitor morphology. Perfusion with 2.5mM NaCN causes a gradual increase in transient amplitude and in diastolic Ca2+. This is followed by cessation of response to field stimulation and going into rigor (critical fall in ATP) associated with elevated Ca2+. Upon reperfusion transients resume but cells can either lose control over calcium and die or show control and resume contractility.

6.2 Aims

The aims of this chapter are therefore;

1) Assess the effects of the cAMP analogues on contractility and Ca^{2+} transients in superfused isolated cardiomyocytes from P14 and adult hearts
2) Determine how metabolic inhibition with NaCN injures those populations of isolated cardiomyocytes
3) Assess whether the organ- level protection previously observed with 8-Br, and to a lesser extent CPT and 6-Bnz, is replicated on the cellular level with the measures of contractility, Ca^{2+} transient amplitude as well as gross measures of cardiomyocyte function such as arrythmia and morphology

6.3 Methods

Cardiomyocytes were obtained for these experiments from adult and P14 rats as outlined in Chapter 2 and Chapter 5, during the experiments discussed in Chapter 5 and so those details are not repeated. The details for how the isolated cardiomyocytes were superfused and their contractility and transients measured are discussed in Section 2.5, but will be briefly reiterated here. The same batch of cardiomyocytes freshly produced from an individual heart were used for one experimental repeat across all conditions studied in order to limit between-arm variation due to unaccounted variation in the isolation process. The total number of hearts used for these experiments are summarised in Table 6-1. For each of these hearts, at each time point assessed, 5 cells/ fields were chosen to study.

Age Group	Contractility Studies	Ca^{2+} Transient Studies
Adult	6	6
P14	6	6

Table 6-1 Numbers of hearts from each age group tested digested and used in examining the response of cardiomyocytes to simulated ischaemia, divided by age group and type of study. Each digested heart was used to provide cardiomyocytes for all experimental treatments in order to limit between- arm variation. For each heart and time point, 5 data points were assessed.

6.3.1 Contractility experiments

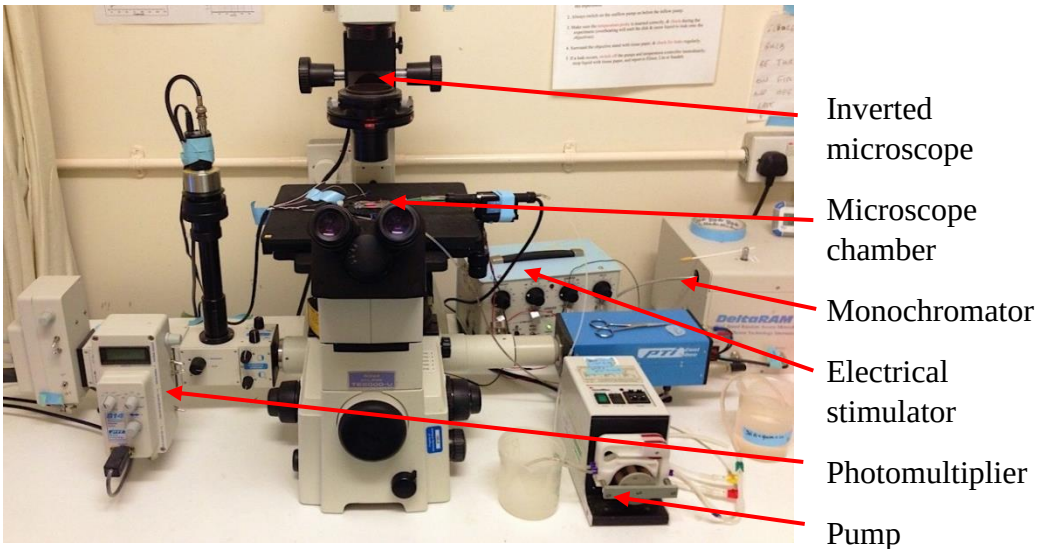


Figure 6-2 Apparatus used for contractility and transient measurements. The microscope chamber is continually perfused with a buffer solution via the pump, and is also intermittently stimulated through peripheral electrical contacts via the electrical stimulator. Shown with light on- used under dim red light with curtain to minimise exposure.

A microscope, Figure 6-2, was used with a custom chamber which allowed for perfusing buffers to be pumped through. A droplet of the cardiomyocyte suspension was placed in the chamber and allowed to settle. A standard HEPES based buffer was used to perfuse the chamber, which was then examined under 10x magnification. A suitable cardiomyocyte or field was selected and the chamber was then stimulated 100V stimulation at 0.2Hz, with a 1 ms delay between pulses. The experimental details are described in Sections 2.4.7.2 and 2.4.7.3. Briefly, an edge tracking device was then used to quantify the contractions observed. Baseline measurements were taken of contractility. Perfusion was then switched to an identical buffer with the addition of 2.5 mM NaCN for 30 minutes, recording of contractility continued, and note taken of the time to the endpoints of contractile arrest, arrhythmia, hypercontracture, and cell death.

In order to process the contractility data, time points at 5 minute intervals from the start of the experiment up to 35 minutes perfusion were selected. The raw contractility data for each of these time points were examined, and three contractile cycles either side of the time point marker were chosen for further analysis. The outputs at these times that represented systole and diastole- the minima and

maxima- were manually identified, and then these converted back from the software's arbitrary units to measurements of cell size. This was accomplished using a previously calibrated relationship for the equipment; a linear relationship existed between the output voltage from the edge tracker and size; it was straightforward to convert from the measured potentials to instantaneous cell size.

These estimates of cell size for diastole and systole could then be combined into a measure of fractional shortening-

$$\frac{\Delta size}{diastolic\ size} = fractional\ shortening$$

The resulting value was then normalised by correcting for the value at baseline (initial value at time 0); it was thus expressed as a ratio of the initial fractional shortening. Comparisons of the data at 5 minutes vs baseline, and at 35 minutes vs. 5 minutes were then made by two-tailed Student's T-tests; this enabled an analysis of the pharmacological action of the cAMP analogues on the cardiomyocyte's physiology (at 5 minutes); and at 35 minutes the data could be examined for a potentially protective effect against hypoxia.

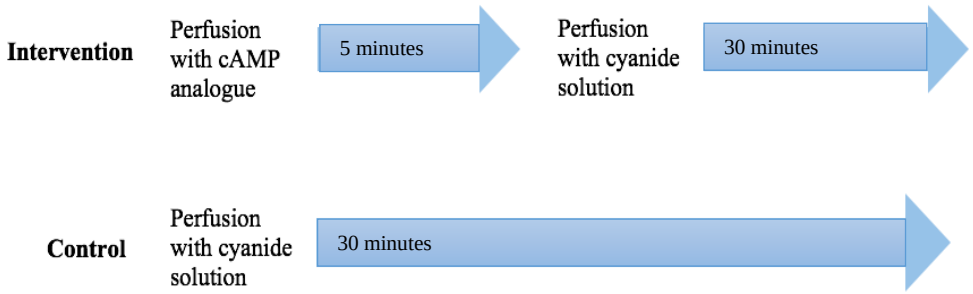


Figure 6-3 Experimental design for contractility studies. Control group cardiomyocytes were perfused with the baseline buffer followed by 30 minutes with the addition of NaCN. Those in the intervention groups had a 5 minute period of perfusion with buffer containing the cAMP analogue before switching to the NaCN containing buffer.

The experimental design is shown in Figure 6-3. Example contractility traces are shown in Figure 6-4, for contraction in standard buffer, in Figure 6-6, the effect of

addition of cyanide, and in Figure 6-5, perfusion with 8-Br before the addition of cyanide.

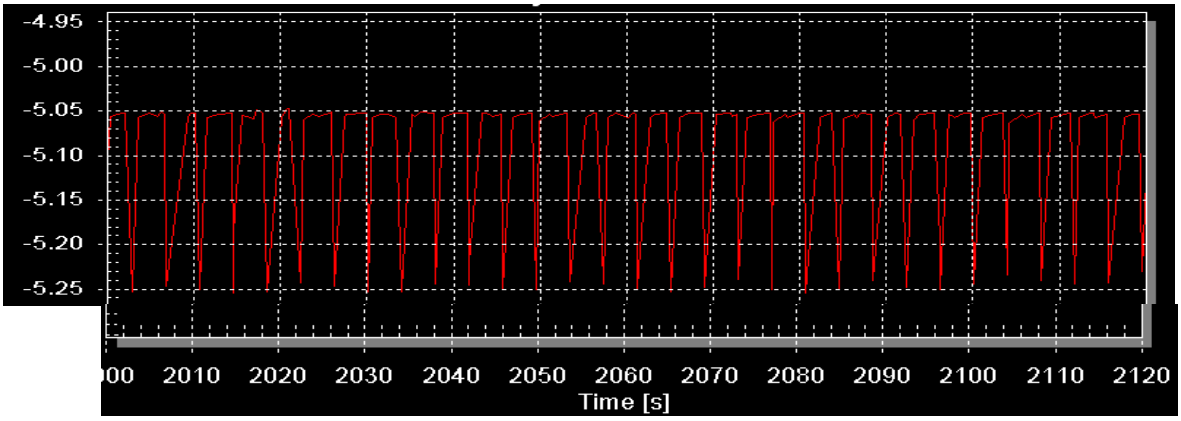


Figure 6-4 Example edge tracking recording of single cardiomyocyte contractions during perfusion with normal buffer at 28^{0}C and stimulated at 0.2Hz

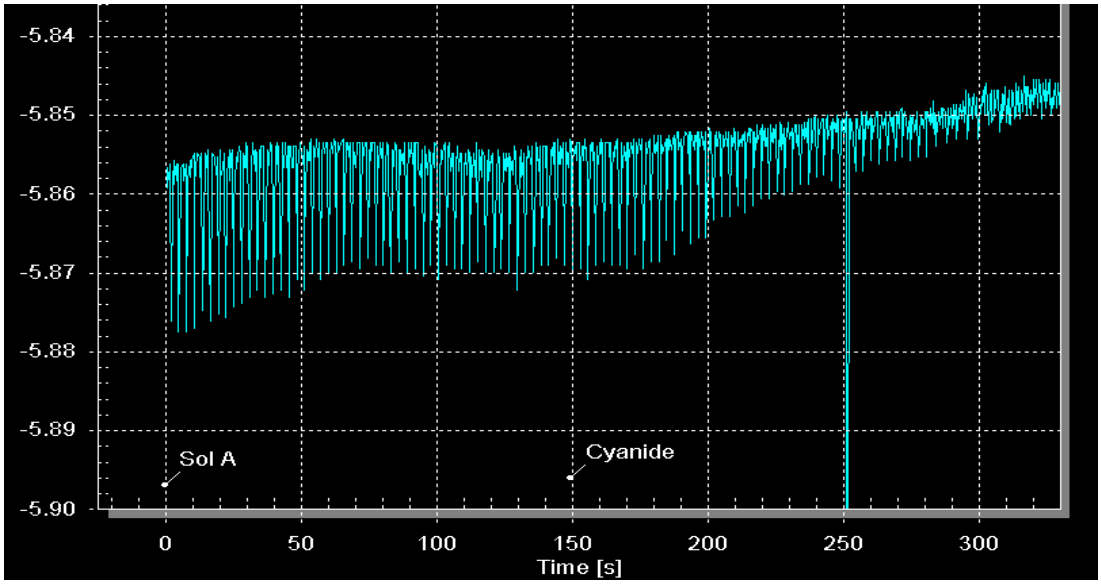


Figure 6-6 Example edge tracking output from PTI of cardiomyocyte contractility during perfusion prior to and with cyanide

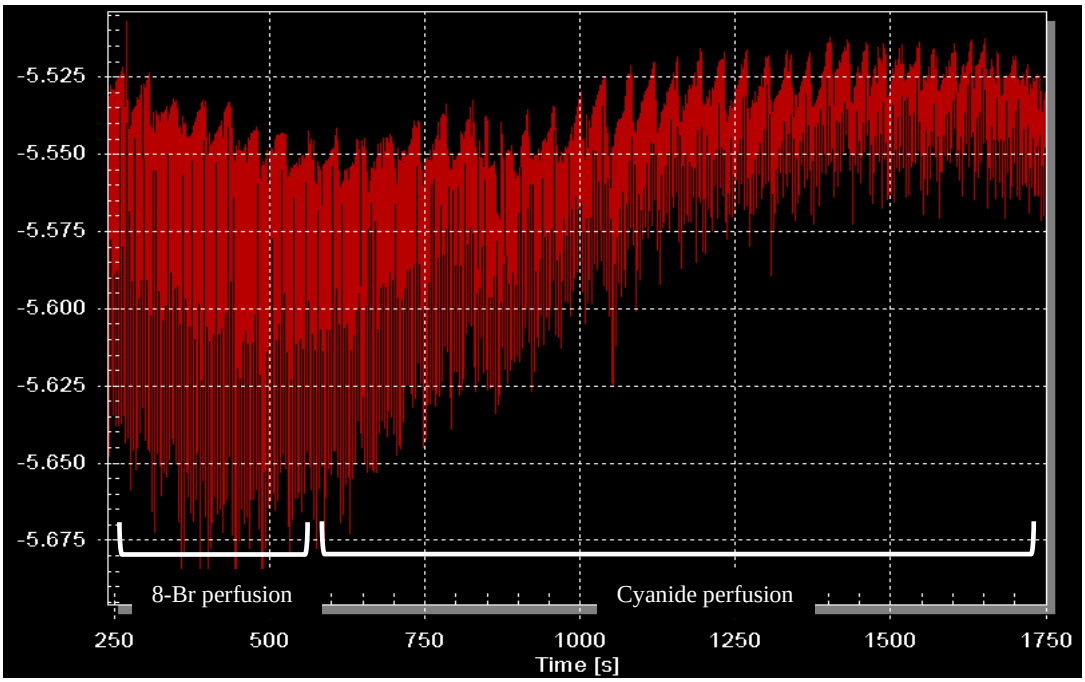


Figure 6-5 Example edge tracking output from PTI of cardiomyocyte contractility during perfusion with 8-Br and subsequently with cyanide

6.3.2 Ca^{2+} transient experiments

These experiments were repeated making measurements of the Ca^{2+} transient with an identical experimental design, methodological detail for which is again described in Chapter 2. Briefly, cardiomyocytes were loaded with the fluorescent dye Fura-2-AM which is cell permeable.

This dye hydrolyses under ultraviolet light to Fura-2, which binds free Ca^{2+} within the cell. The bound and unbound forms of Fura have two differing excitation wavelengths (340 nm vs 380 nm bound and unbound respectively) and so therefore the ratio of these allows estimation of the proportion of free Ca^{2+} in the cardiomyocyte. This ratio was measured continuously throughout the periods of perfusion with the baseline buffer, the cAMP analogues, and with NaCN containing buffer. An example trace is shown in Figure 6-7.

The experiments upon isolated cardiomyocytes examining their contractile behaviour and calcium transients had two phases; the first, was their stimulated behaviour under the influence of perfused cAMP analogues. The second was an examination of their behaviour following simulated hypoxia. I therefore analyse these two phases separately, as a purely pharmacodynamic phenomenon to begin with, followed by the pathophysiology of histotoxic hypoxia.

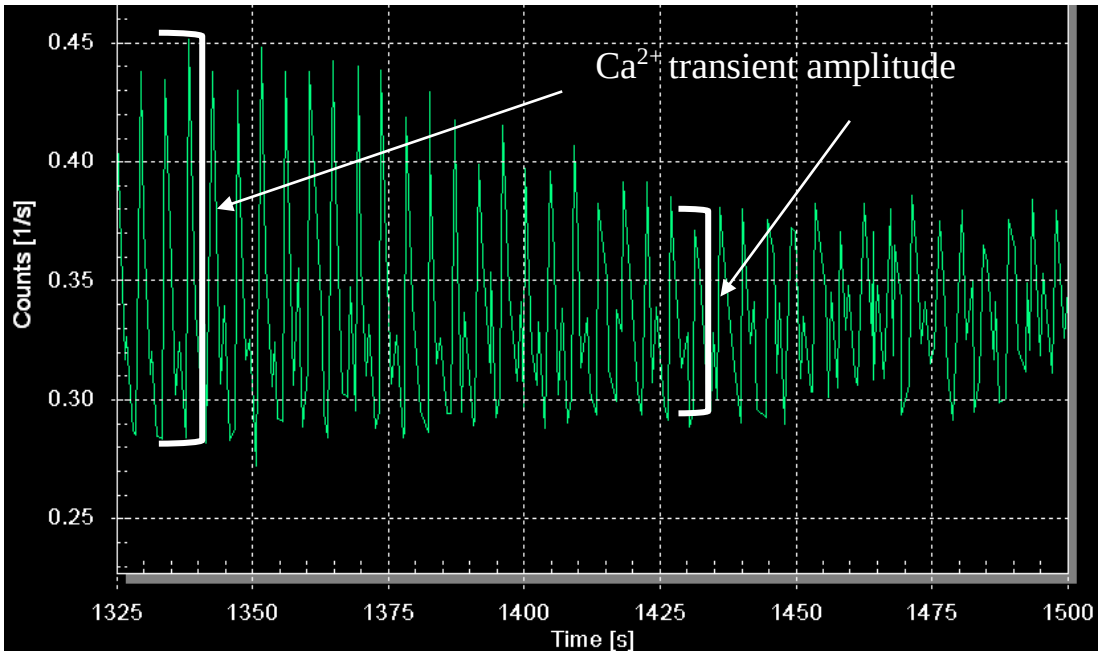


Figure 6-7 Example Ca^{2+} transient trace from a cardiomyocyte perfused with standard buffer before switching to buffer containing NaCN.

6.4 Results

6.4.1 Influence of cAMP analogues on isolated cardiomyocyte contractility & Ca^{2+}Transient in the absence of Simulated Hypoxia

6.4.1.1 The response of adult cardiomyocytes

Cardiomyocytes showed a positive inotropic response when perfused with analogues of cAMP. Of these, perfusion with 8-Br generated the greatest magnitude of response; a just over two-fold increase in contractility (2.01±0.050, Figure 6-8).

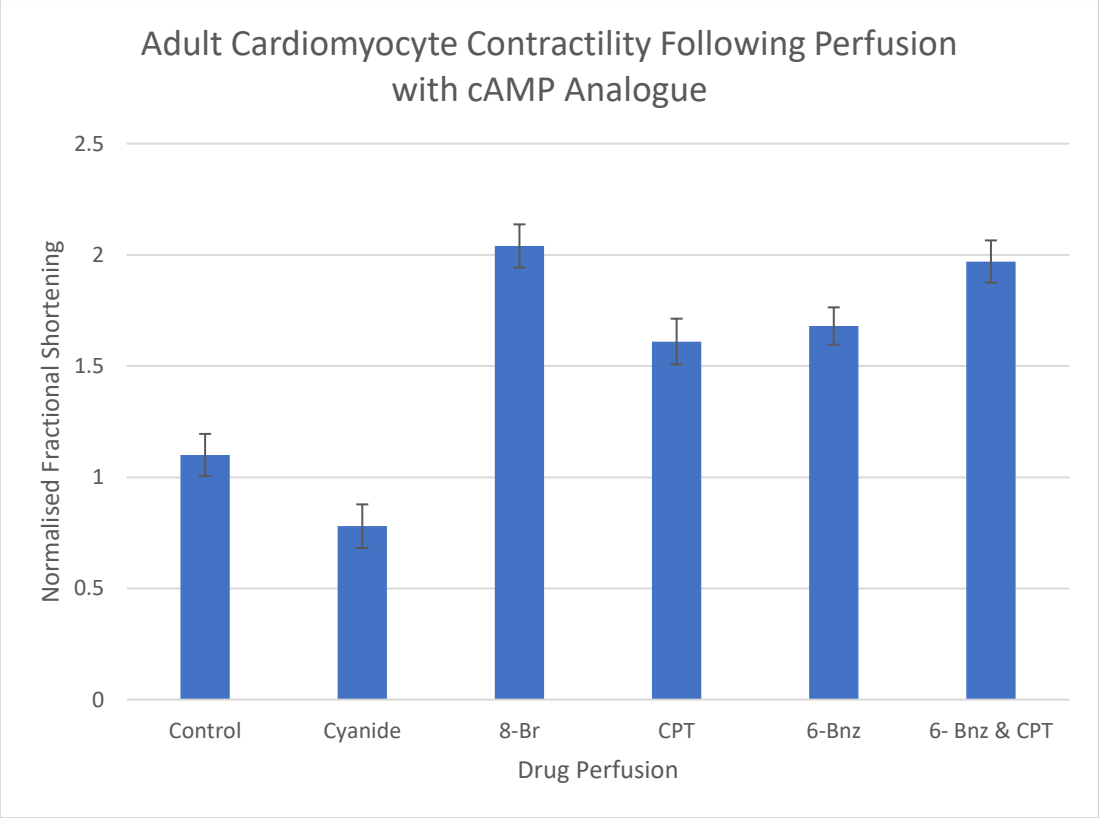


Figure 6-8 Change to Adult Isolated Cardiomyocyte Contractility following perfusion with cAMP analogue or cyanide. Change to fractional shortening after 5 minutes perfusion in each experimental arm in adult cardiomyocytes; normalised against baseline contractility. Data- mean ±SEM. All data points statistically significant by two-tailed unpaired T-test at p< 0.05 vs. control perfusion; n=6 hearts.

The selective agonists of PKA and Epac, 6-Bnz and CPT, individually also elicited a positive inotropic response. 6-Bnz perfusion caused a 63% increase in contractility, whilst CPT generated a similar reaction, increasing normalised contractile shortening by 60%. Expressed as a proportion of the response to either of these agents, 8-Br

elicited a 40.9% greater contractile response vs. CPT and 37.4% greater response vs. 6-Bnz.

Similarly, each cAMP analogue showed an induction of a significant increase in the magnitude of the calcium transient. This peaked in amplitude after five minutes of perfusion; the peak in size was to an almost identical degree with any of the cAMP analogues, at just over two- fold; a mean of 2.14 fold for 8-Br, with no significant differences to CPT or 6-Bnz (Figure 6-12, Figure 6-13, shown with data for results following cyanide perfusion).

6.4.1.2 The response of P14 myocytes

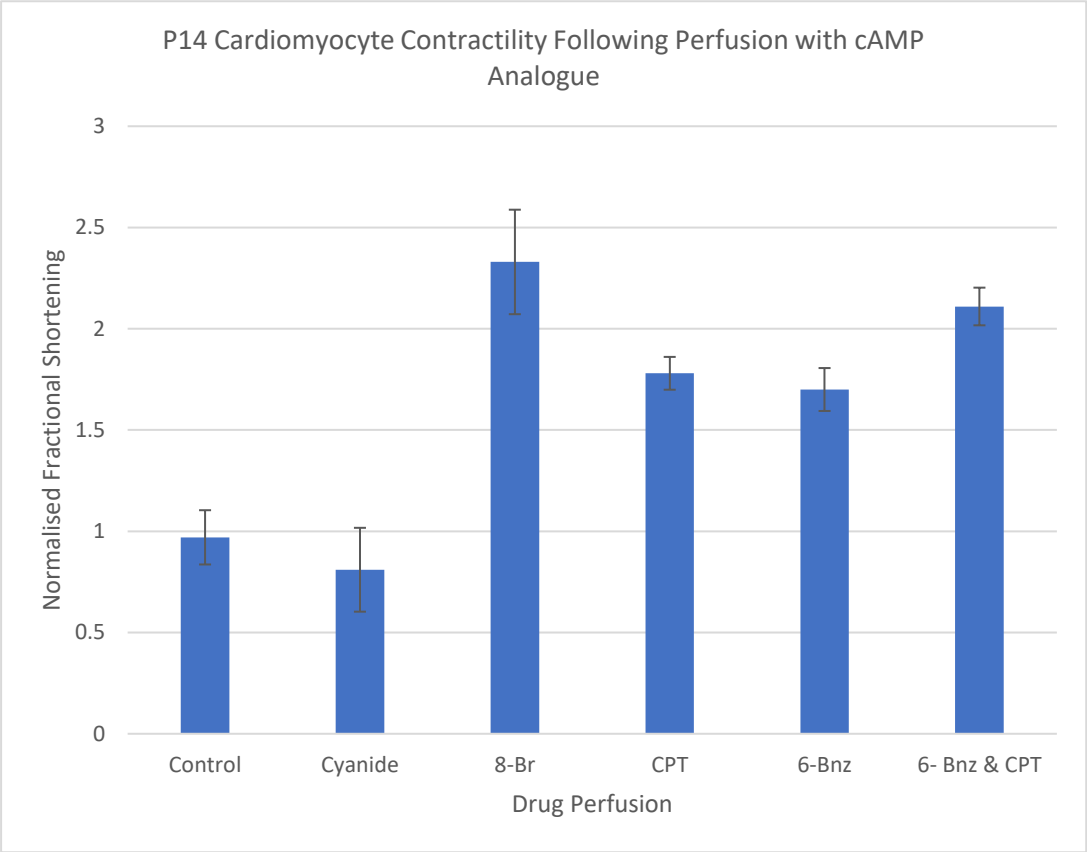


Figure 6-9 Change to P14 Cardiomyocyte Contractility following perfusion with cAMP analogue or cyanide.

Change to fractional shortening after 5 minutes perfusion in each experimental arm in adult cardiomyocytes; normalised against baseline contractility. Data- mean ±SEM. All data points statistically significant by two-tailed unpaired T-test at p< 0.05 vs. control perfusion other than cyanide arm; n=6 hearts.

Immature cardiomyocytes also showed a positive inotropic response to 8-Br perfusion. There was a mean increase of 2.33- fold ± 0.26 after 5 minutes perfusion. The effect of the single target agonists was smaller, but still markedly elevated compared to control. CPT caused a 1.78±0.081x increase vs baseline, and 6- Bnz 1.70±0.11x. The response of combined perfusion with 6-Bnz & CPT together was comparable to that of 8-Br, at 2.11±0.093x.

Similar results to the adult were found for response of the Ca^{2+} transient to drug perfusion. Each cAMP analogue showed a significant increase in the magnitude of this response. This peaked in amplitude at the five minute sampling point; the peak in size similar across the cAMP analogues, at almost 2.5 fold; a mean of 2.48 ± 0.11 fold for 8-Br mirroring the change in contractility, with a similar pattern of response

for CPT and 6-Bnz (Figure 6-17, Figure 6-18, shown with data for results following cyanide perfusion).

6.4.2 Pre-treatment with cAMP analogues and simulated hypoxia

6.4.2.1 Adult Cardiomyocytes

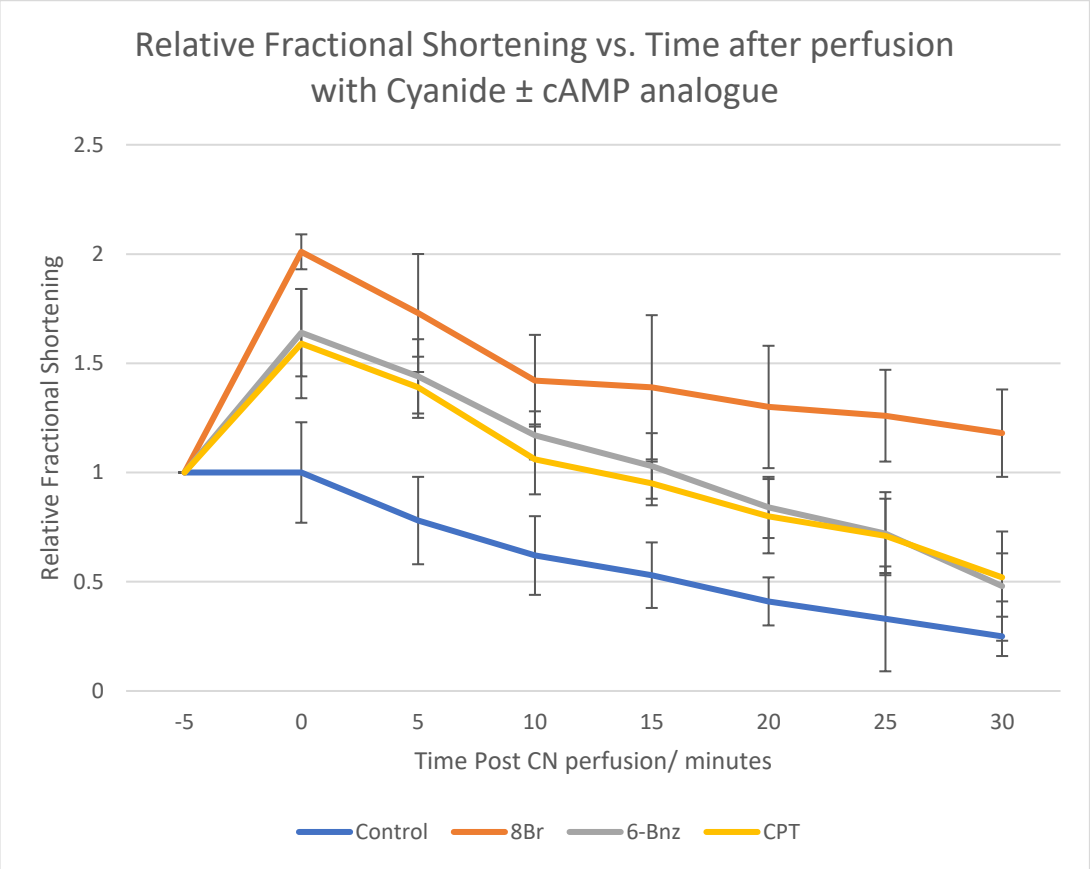


Figure 6-10. Relative fractional shortening of Adult Cardiomyocyte vs time after perfusion with cAMP analogue + cyanide

Prior perfusion with cAMP analogue (as noted) or unmodified buffer ('cyanide'). Cyanide added to buffer after 5 minutes perfusion (T=0). Data shown as mean ± SE; n=6 hearts.

As shown in Figure 6-10, perfusion with a sodium- cyanide containing buffer causes a steady decrease in contractility; a cell typically being reduced in fractional shortening to 21.2% ± 9.0% of the baseline value after 30 minutes perfusion. Perfusion with a buffer containing either 10 µM 6-Bnz or CPT as well as the sodium cyanide provides a transient amelioration of this effect, but at the end of the 30 minute perfusion period the contractile function of these cells is indistinguishable from those containing cyanide alone. However, in the experiments in which cells were perfused either with 8-Br, or with a combination of 6-Bnz and CPT as well as

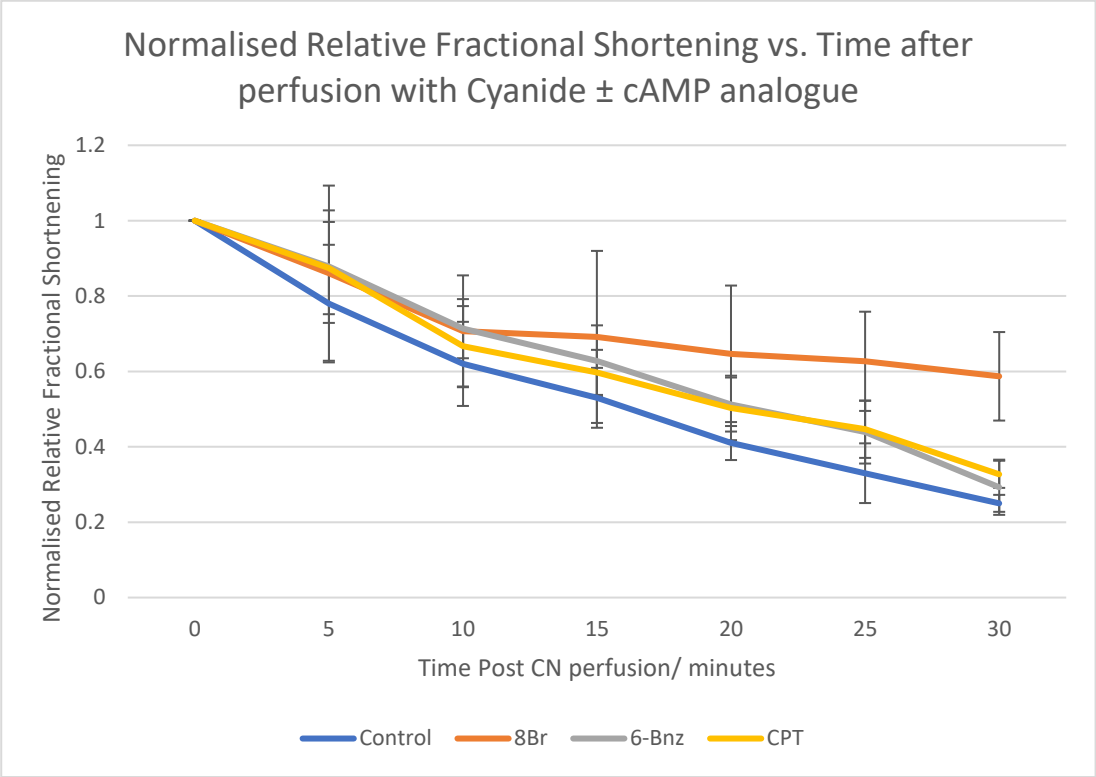


Figure 6-11 Normalised relative fractional shortening of adult cardiomyocytes vs time after perfusion with cAMP analogue + cyanide. Normalised to contractility at the point of addition of cyanide to perfusing buffer. Data shown as mean ± SE; n=6 hearts.

with cyanide, there was a sustained amelioration of the functional impairment seen with cyanide alone. As compared to the pre-perfusion values, perfusion with 8-Br led to a normalised contractility value after 30 minutes of 1.08±0.199; this is higher than at baseline despite concurrent perfusion with cyanide, such that at the end of the experiment those cells perfused with 8-Br and cyanide had a contractility in excess of five-fold those perfused with cyanide. A similar degree of rescue from the effects of perfusion with cyanide was seen with the addition of the combination of 6-Bnz and CPT; resulting in a relative contractility at 30 minutes perfusion of 0.850±0.0018, showing a synergistic characteristic resulting from the simultaneous perfusion of these two agents.

The Ca^{2+} transient data follows a similar pattern to the contractility, with 6-Bnz and CPT following a relatively linear pattern of decline during washout and CN perfusion after their peak during drug perfusion (

Figure 6-12). However, the results for 8-Br are qualitatively different. Here, throughout perfusion with NaCN, the transient amplitude remains greater than the baseline value, demonstrating a lasting effect of the drug despite washout and simulated hypoxia.

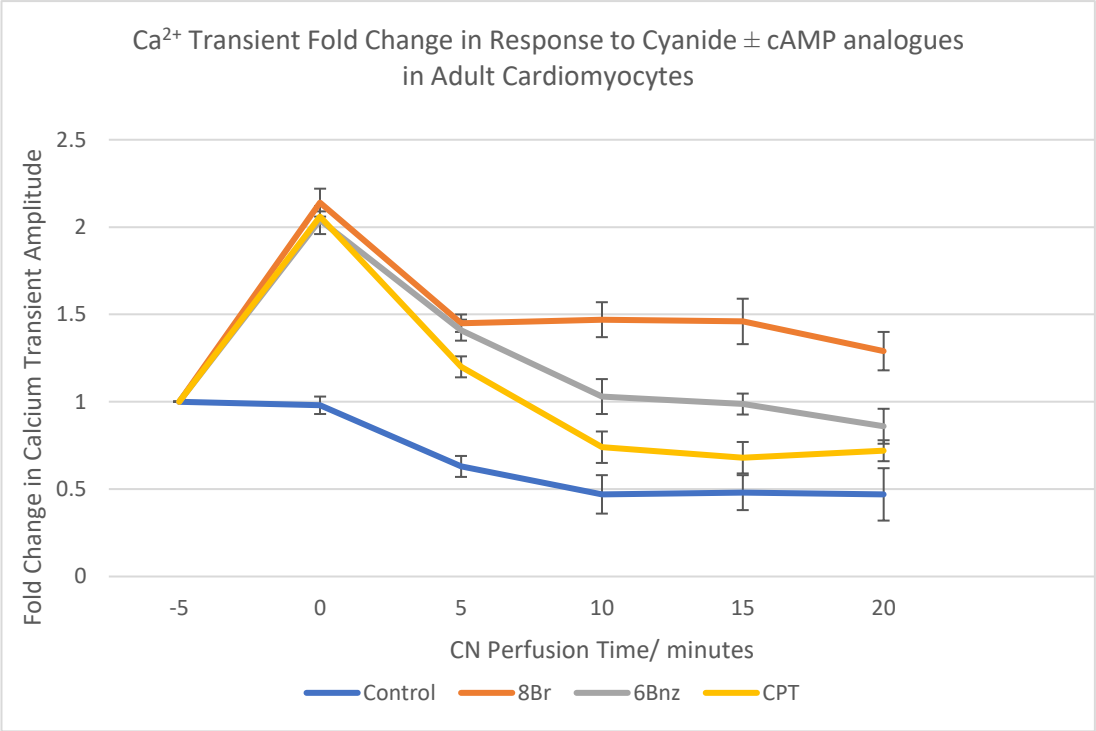


Figure 6-12. Mean peak amplitude of calcium transient in adult cardiomyocytes prior to and following perfusion with cAMP analogue and cyanide.Normalised to the initial value of amplitude at the start of control perfusion (T=-5), and after cyanide added to perfusing buffer at 0 minutes. Control (cyanide) vs buffer containing each cAMP analogue. Control= no addition to buffer other than cyanide. Data points represent mean +/- SEM. N=6 hearts.

The transient results were was then normalised to the maximum value measured during perfusion with the cAMP analogue (or baseline for the control). Interestingly, even after the drug had been washed out and throughout the perfusion with NaCN, the cells perfused with 8-Br have a normalised calcium transient amplitude significantly greater than that of the controls well after the drug has been removed and corrected for the peak during drug perfusion (Figure 6-13).

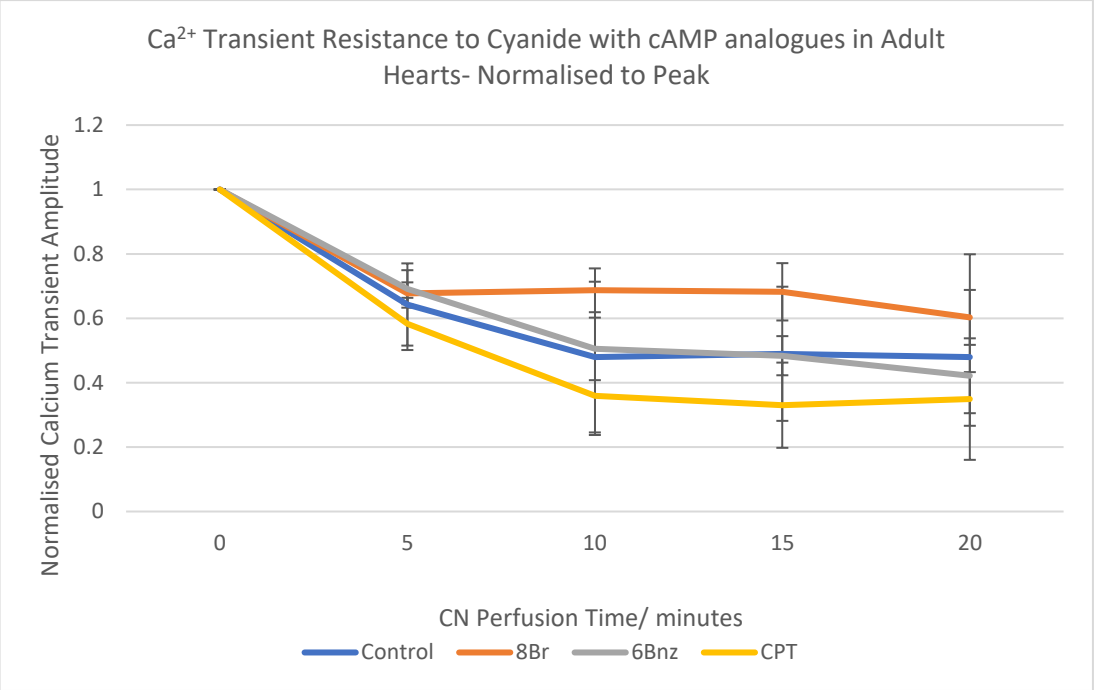


Figure 6-13 Normalised amplitude of calcium transient in adult cardiomyocytes following perfusion with cAMP analogue and cyanide.

Normalised to the initial value at time points at the start of cyanide perfusion (T=0) following 5 minutes of preceding perfusion with cAMP analogue or control. Underlying data identical to previous figure. Control= no addition to buffer other than cyanide. Data points represent mean ±SEM. N=6 hearts.

The time taken for cells to reach a variety of endpoints (time to arrhythmia, to contractile cessation, to cell death, and to hypercontraction) under perfusion with each drug was also measured Figure 6-14. For each of these end points, perfusion with a cyanide- containing buffer significantly shortened the time that end-point. This was also true for 8-Br perfusion for time to arrhythmia and time to hypercontraction; however no significant difference to control was seen for time to cell death. Perfusion with CPT nor 6-Bnz did not lead to a shortening of the time to any of these outcomes, although there was an observed trend for a pro-arrhythmic propensity.

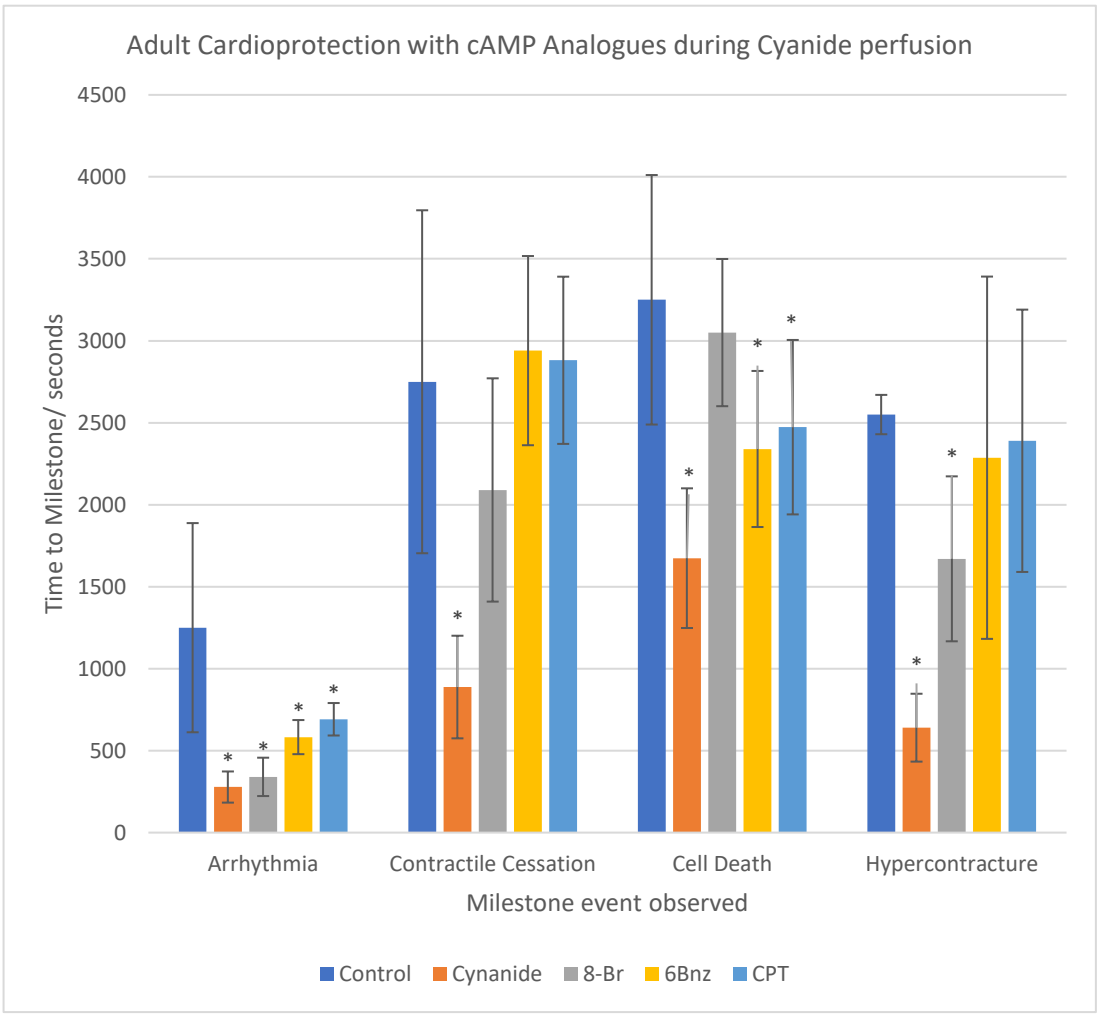


*Figure 6-14 Time taken to reach pathological endpoints for adult cardiomyocytes perfused with cyanide ± a cAMP analogue. Data points represent mean ±SEM. *= p<0.05 vs. control (HEPES buffer perfusion alone). Statistical testing by 2-tailed unpaired T-test. N=6 hearts. 5 cardiomyocytes per endpoint per heart studied.*

6.4.2.2 P14 Cardiomyocytes

In the P14 group, a similar pattern of decline in contractility following simulated hypoxia with NaCN is seen to that in the adult (Figure 6-16 & Figure 6-15). All drug intervention groups demonstrate a decline in their contractility. However, by 30

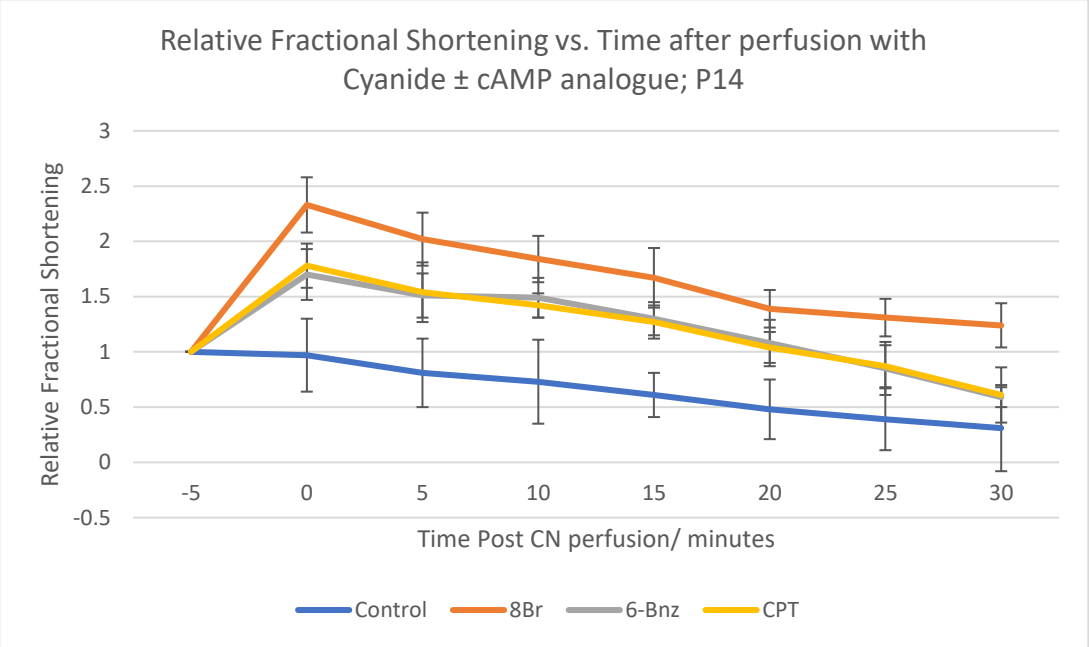


Figure 6-16 Relative fractional shortening of P14 Cardiomyocyte vs time after perfusion with cAMP analogue + cyanide.

Prior perfusion with cAMP analogue (as noted) or unmodified buffer ('cyanide'). Cyanide added to buffer after 5 minutes perfusion (T=0). Data shown as mean ± SE; n=6 hearts.

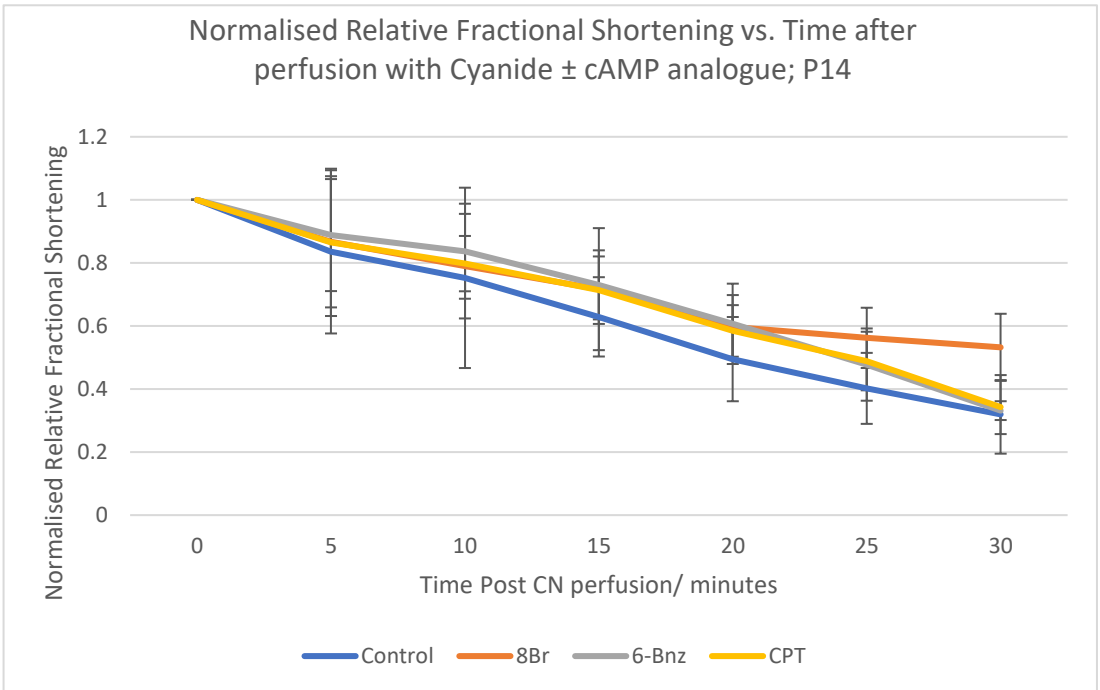


Figure 6-15 Normalised relative fractional shortening P14 cardiomyocytes vs time after perfusion with cAMP analogue + cyanide. Normalised to contractility at the point of addition of cyanide to perfusing buffer. Data shown as mean ± SE; n=6 hearts.

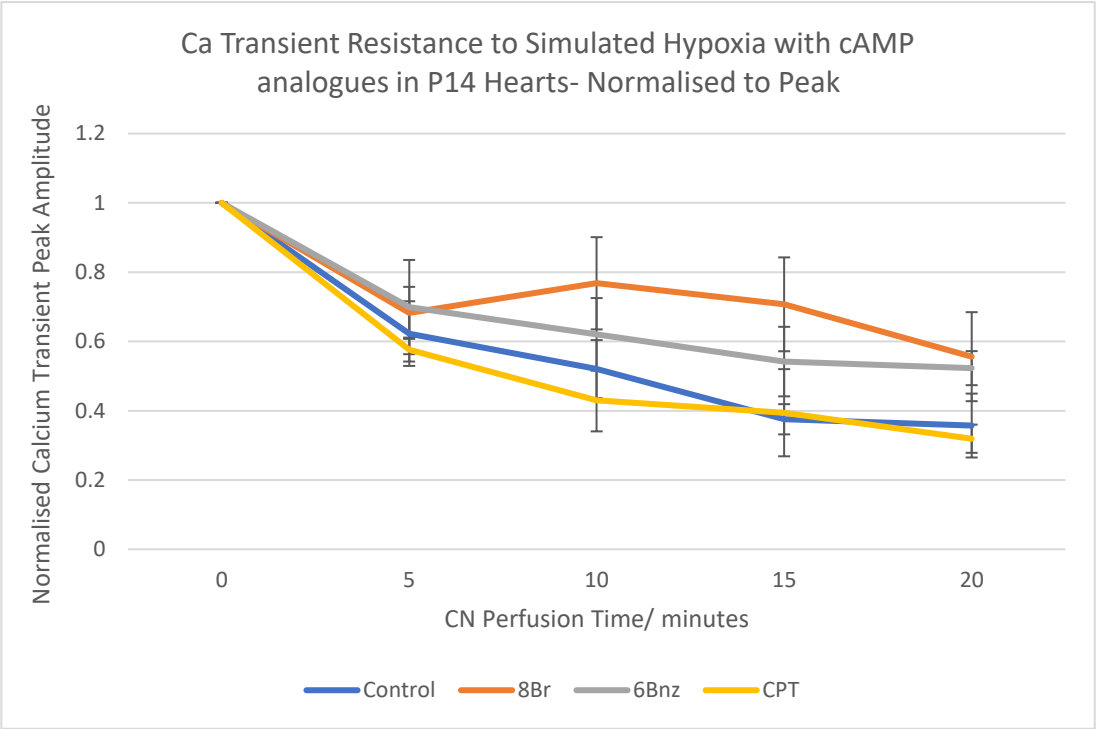


Figure 6-18 Mean peak amplitude of calcium transient in P14 cardiomyocytes prior to and following perfusion with cAMP analogue and cyanide.

Normalised to the initial value of amplitude at the start of control perfusion (T=-5), and after cyanide added to perfusing buffer at 0 minutes. Control (cyanide) vs buffer containing each cAMP analogue. Control= no addition to buffer other than cyanide. Data points represent mean +/- SEM. N=6 hearts.

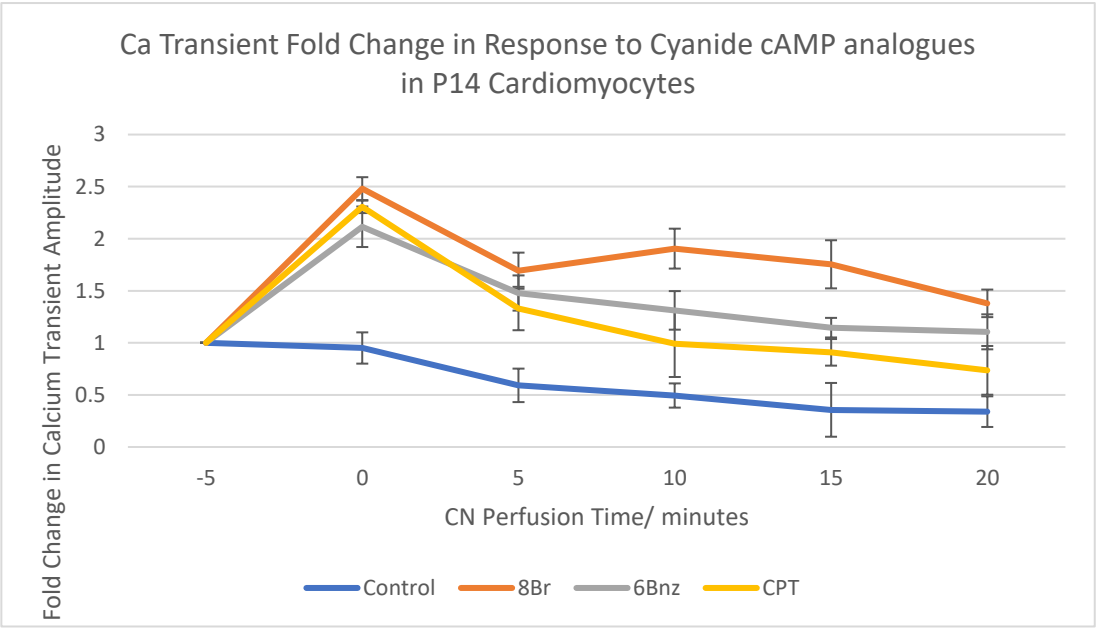


Figure 6-17 Normalised amplitude of calcium transient in adult cardiomyocytes following perfusion with cAMP analogue and cyanide.

Normalised to the initial value at time points at the start of cyanide perfusion (T=0) following 5 minutes of preceding perfusion with cAMP analogue or control. Underlying data identical to previous figure. Control= no addition to buffer other than cyanide. Data points represent mean ±SEM. N=6 hearts.

minutes of perfusion with NaCN, only the 8-Br group is improved vs. control, and

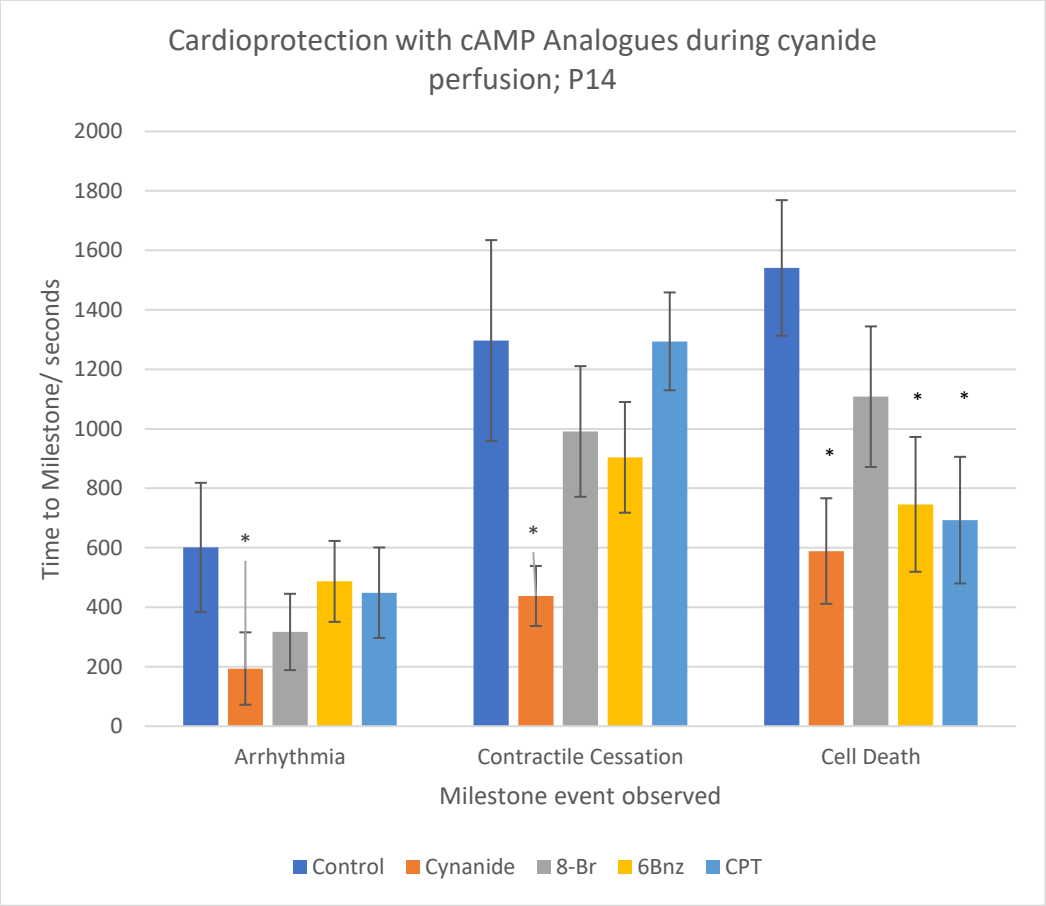


*Figure 6-19 Time taken to reach pathological endpoints for P14 cardiomyocytes perfused with cyanide ± a cAMP analogue. Data points represent mean ±SEM. *= p<0.05 vs. control (HEPES buffer perfusion alone). Statistical testing by 2-tailed unpaired T-test. N=6 hearts. 5 cardiomyocytes per endpoint per heart studied.*

indeed as in the adult it remained improved on the baseline contractility despite the simulated hypoxia. Perfusion with CPT or 6-Bnz did not show this effect, and they were not significantly different to control at 30 minutes.

The data showing the response of the Ca^{2+} transient in the P14 cardiomyocyte to stimulation with cAMP analogues prior to perfusion with cyanide are shown in Figure 6-17 (raw data) and Figure 6-18 (normalised to peak amplitude just prior to cyanide perfusion. As for the equivalent adult data, these show as similar trend as for the contractility results; 8-Br, CPT and 6-Bnz all cause at least a 2.1 fold increase in systolic Ca^{2+} levels, which then falls away as perfusion with cyanide begins from t=0 onwards. Only 8-Br maintains a positive, maintained effect upon the Ca^{2+} transient throughout the period studied, although CPT and 6- Bnz do seem to have an intermediate effect between the preservation seen with 8-Br and the reduction to 39% of baseline seen in the control group. Considering the endpoints of cell death,

arrythmia and contractile cessation (Figure 6-19), perfusion with NaCN significantly shortened the time to observation. (Hypercontracture could not be reliably assessed in the P14 group due to the rounded & stellate initial appearances of the cardiomyocytes). 8-Br, CPT, and 6-Bnz all improved the time to contractile cessation to the point they were comparable to control. However, for time to arrhythmia, there was no significant difference to control. Again, only 8-Br produced a protective effect against time to cell death (1108s vs 509s); there was no protection seen in the individual actions of 6-Bnz or CPT in this model of hypoxia.

6.5 Discussion

Through this work, I hope to have demonstrated that;

- Perfusion of both adult and immature cardiomyocytes with PKA & Epac agonists is associated with an inotropic effect
- This effect correlates with changes to the peak Ca^{2+} transient
- Perfusion with cyanide is an effective model of hypoxia in the isolated cardiomyocyte, showing reductions in Ca^{2+} transients and contractility
- There is no great difference in vulnerability between the P14 and adult derived cardiomyocytes when exposed to this method of metabolic inhibition
- Epac & PKA agonists ameliorate loss of contractility and calcium transients during histotoxic hypoxia in both adult & P14 cardiomyocytes
- Synergistic activation of PKA & Epac lengthens the time to cell death during cyanide perfusion but may be pro- arrythmogenic

6.5.1 Perfusion of cardiomyocytes with cAMP analogues demonstrates physiological response of inotropy

The results of baseline perfusion of isolated cardiomyocytes with any of the cAMP analogues used demonstrate that they all trigger an increase in calcium transient amplitude, as well as an increase in contractility of the cardiomyocytes originating from both P14 and adult rats. These effects are likely mediated through PKA & EPAC signalling through the L-type calcium channel (Fujita, Umemura et al. 2016, Lezoualc'h, Fazal et al. 2016), and through phospholamban, which collectively enhance calcium entry into the cell and aid in the reuptake at the end of systole.

This concurs with other observations of these agents performed in whole heart preparations in Chapter 4, and those such as Khaliulin & colleagues (Harmati, Banyasz et al. 2011, Khaliulin, Bond et al. 2017), that these agents do act as thought as

agonists along the β-AR signalling pathway. These effects were also increased with perfusion with 8-Br, versus either 6-Bnz or CPT alone, implying that 6-Bnz and CPT acted independently through effector pathways which are both activated during perfusion with 8-Br resulting in maximal contractile response.

These will ultimately be acting to increase the increase in calcium- induced calcium release during excitation- contraction coupling (Tohse, Seki et al. 2004). There are multiple routes by which this might be accomplished, through targets such as phospholamban and the ryanodine receptor; these targets are well established for signalling through PKA, but less so through EPAC (Oestreich, Malik et al. 2009, Pereira, Ruiz-Hurtado et al. 2012).

6.5.2 Simulated hypoxia induces changes in contractility and Ca^{2+} transients

Perfusion of the cardiomyocyte suspension with a buffer containing cyanide induces a significant reduction in both calcium transient amplitude, and contractility of the cells in both the P14 and adult groups. The proposed mechanism of action of cyanide in this context is as an inhibitor of aerobic respiration via mitochondrial electron transport, specifically by inhibiting cytochrome aa_3 in the mitochondrial complex IV (Murphy and Steenbergen 2008). Therefore, this is a model of the effects of simulated hypoxia on isolated mitochondria, and so a valid way of studying the influence of the cAMP analogues on cardiomyocytes subjected to this form of hypoxia. This is not synonymous with ischaemia; that would require a perfusate deprived of all sources of energy, with not continuous flow to remove metabolic byproducts.

6.5.3 EPAC & PKA agonists ameliorate loss of contractility and calcium transients during histotoxic hypoxia

After exposure to cyanide, cardiomyocyte contractility decreases steadily over the remaining 30 minutes of the experimental procedure. Figure 6-10 & Figure 6-16

demonstrate that all of the cAMP analogues used for these experiments ameliorate this effect to a greater or lesser extent. 8-Br provided the most effective protection against this functional injury; with 5 minutes prior perfusion, there was no difference between the contractile function after 30 minutes exposure to cyanide and the baseline contractility which was significant with a p-value <0.001. CPT and 6-Bnz also had a protective effect but to a lesser degree; contractility fell to 51 and 49% of baseline respectively. The control arm, for comparison, fell to a mean value of 22% of baseline contractility. These effects are not due to ongoing direct stimulation by the agonists; after 30 minutes of continual perfusion, the drugs will have been washed from the system, and so the prior perfusion with the drugs must have ongoing effect upon the internal milieu of these cardiomyocytes.

A similar effect is seen when considering the calcium transient. Perfusion with 8-Br, with 6-Bnz, or with CPT leads to a maintained & significant elevation of the calcium transient concentration following perfusion with a cyanide- containing buffer. However, this effect is most strongly noticed with 8-Br, the effects of the single agonists being only a fraction of that seen with 8-Br.

That 6-Bnz and CPT only provide a fraction of the protection seen with 8-Br, implies that (assuming roughly equal efficacy as agonists) they act via independent signalling pathways and that the additional effect from 8-Br results from co-incident activation of these pathways. That simultaneous administration of 6-Bnz and CPT, as in Figure 6-8 & Figure 6-9, show a similar combined effect as perfusion with 8-Br alone, implies that their mechanisms seem to be synergistic. An end-point for the inotropic action of β-AR stimulation has been thought to be through cAMP triggered RyR phosphorylation (Bers 2002); it may be that both PKA and EPAC act to effect this phosphorylation, possibly on differing sites.

6.5.4 EPAC & PKA agonists cause a reduction in the likelihood of arrhythmia, as well as time to hypercontraction and cell death following cyanide perfusion

The experimental arm least prone to arrhythmogenesis is that with no intervention. This is unsurprising, as there is no intervention to predispose towards metabolic, electrical or contractile abnormality.

The onset of arrythmia in cardiomyocytes perfused with cyanide without drug treatment was the quickest compared to drug treated myocytes. Cyanide perfusion (metabolic inhibition) inhibits ATP production, and this results in loss of SERCA function, resulting in elevation and eventual overload in intracellular Ca^{2+} (Venkataraman, Holcomb et al. 2012). This overload will result in arrhythmogenesis, explaining why CN perfusion results in this observation.

Treatment with 8-Br prior to administration of CN had the next shortest time followed by those containing CPT. This may be because SERCA is a downstream target of EPAC signalling, increasing its activity whilst ATP remains available and thus delaying the onset of calcium overload. However, this would need direct measurement of SERCA activity in this context to confirm.

A similar consequence of calcium overload is that of contractile cessation and hypercontraction of the cardiomyocyte (Ruiz-Meana, Garcia-Dorado et al. 1999, Marks 2003). This is because of the failure to restore normal levels of intracellular calcium by ATP-deprived SERCA following depolarisation and contraction, and so the cardiomyocyte fails to relax. In addition, ATP is required to release the actin-myosin interaction, so leading to a further cause of hypercontraction. Those cells given 8-Br or CPT prior to cyanide perfusion had a significantly longer time to hypercontraction and to cessation of contraction. Again this is likely to be due to enhanced SERCA activity, via phosphorylation of PLB downstream of cAMP signalling. It is interesting to note that 8-Br prolongs this time less than CPT. This may be due to the differing affinities and efficacies of the two agents (Rehmann, Schwede et al. 2003), but it is also possible that the PKA activity related to 8-Br has other negative regulatory activity on this system.

Cardiomyocyte death took longest in the control group and was quickest in the group perfused with cyanide alone. This effect was almost completely abolished by prior perfusion with 8Br, and to a lesser extent by CPT. The mode of death for all of the cells studied in these experiments was observed to be apoptotic; a final common pathway for apoptotic cell death involves opening of the MPTP and release of cytochrome c from the mitochondrion (Suzuki, Yokoyama et al. 2010), prior to the activation of the effector caspases. As will be discussed in the experiments on mitochondrial isolation, it is possible that 8-Br and CPT are reducing the likelihood of pore opening and so prolonging the time until an injured cell commits to apoptosis. However, the prior literature is equivocal on the impact of EPAC and PKA signalling on apoptosis , with both being reported to have both pro- (Insel, Zhang et al. 2012, Okumura, Fujita et al. 2014) and anti- apoptotic effects (Kwak, Park et al. 2008, Mangmool, Hemplueksa et al. 2015). It is likely that the context, time- course, and duration of the signalling stimulus is critical to determining the outcome. Further biochemical studies on effectors of apoptosis in cardiomyocytes briefly exposed to these cAMP analogues in the context of this model of injury would be illuminating.

The next chapter examines the direct role of these signalling pathways on mitochondrial permeability transition and is aimed at providing a mechanistic insight into how amelioration from ischaemia/ reperfusion injury may be occurring.

7 The effects of cAMP/PKA/Epac signalling on MPTP opening

7.1 Introduction

In the previous chapters I have shown that both activity at the β- adrenergic receptor and at intermediaries downstream confer protection of the whole adult and immature Langendorff perfused heart to ischaemia and reperfusion injury; and that isolated cardiomyocytes, divorced from whole organ structure and a plethora of other cell types in the heart, may also be protected by stimulating this signalling pathway.

Ischaemia and reperfusion injury are mediated by Ca^{2+} loading and generation of ROS which in turn trigger opening of MPTP leading to death by necrosis and apoptosis (See Introduction, Role of MPTP in I/R Injury, 1.3.3). Therefore, prevention of pore opening is a strategy aimed at ameliorating the damage to the heart exposed to ischaemia and reperfusion injury. The role and even the formation of the MPTP under physiological conditions is uncertain, however. Some have argued that under the non-pathological state it plays a key role in mitochondrial Ca^{2+} homeostasis (Ichas and Mazat 1998), whereas others have disputed whether the components even form a complex without the stimulus for permeability transition (Introduction 1.3.2).

The opening of the MPTP and swelling of the mitochondria lead to loss of membrane potential and electron transport chain uncoupling. Each of these events may be studied in order to assess the state of the mitochondria, and from there the effectiveness of any potentially protective intervention. There are viable experimental approaches to assessing each of these elements. Bernardi *et al.* describe an overview of the experimental approaches to this in *Mitochondria and Cell Death* (Bernardi, Scorrano et al. 1999). The most straightforward and direct approach to monitoring MPTP opening was to study spectrophotometrically the swelling induced by addition of Ca^{2+} to a suspension of isolated cardiac mitochondria. This approach allowed perfusion of the heart with an agent of choice prior to isolation, and then an assessment of the resulting mitochondrial biology independent of their host tissue. Therefore, this experimental approach has been utilised to identify direct effects of stimulating cAMP/PKA/Epac signalling pathways on MPTP.

7.2 Aims

The experiments described here had several purposes;

1) To obtain functionally reliable mitochondria from both adult and immature hearts and to establish MPTP opening by elevated Ca^{2+}.
2) To investigate the effect of cAMP/PKA/Epac signalling pathways on MPTP opening under normal physiological conditions and during I/R.
3) To elucidate the effect of cAMP/PKA/Epac signalling pathways in modulating MPTP sensitivity to Ca^{2+} following I/R.

7.3 Methods

The procedure for mitochondrial isolation and swelling assays are detailed in Materials and Methods, Chapter 2, along with the details of the buffers used. In brief, however, hearts were extracted from adult and immature (14 day old) rats by concussion, cervical dislocation and then dissection through sternotomy. These hearts were rapidly immersed in cold (4 °C) Krebs- Henseleit buffer, and the superior aspect explored to identify the aorta. The heart was then cannulated in the Langendorff mode. Exclusion criteria for hearts perfused *ex vivo* are described in Section 4.3.2 and were implemented identically for these experiments.

The experimental protocols used during heart perfusion are described in Section 7.3.1. Following these protocols, the hearts were removed from the aortic cannulae and placed into chilled buffer. They were then homogenized used a Polytron Kinematica probe, and the resulting suspension centrifuged at 2000g to separate the cell debris.

The supernatant from that step was then removed, and centrifuged again at 10,000g for two iterations of five minutes, with the supernatant removed and pellet resuspended each time. The resulting suspension was considered to be a preparation of isolated mitochondria.

These mitochondria would be of variable concentration due to the differing masses of the hearts used initially, and the varying proportion of mitochondria within those

hearts. The concentration was then estimated through a Bradford essay, and this information used to normalise at 0.2 mg/ml in the mitochondrial swelling buffer.

The swelling of these mitochondria in response to Ca^{2+} was then assessed in a spectrophotometer (Evolution 201, ThermoScientific, Waltham, United States) at 520 nm. A baseline recording at 37 °C was made, before the addition of Ca^{2+} to the solution. For the experiments in Sections 7.4.1 and 7.4.2, 3 mM was used as is conventional in our group working with non- ischaemic mitochondria to increase the sensitivity of the response. For those in 7.4.3, as the mitochondria were obtained in ischaemic conditions, the Ca^{2+} concentration used was 1 mM to ensure the response detected was within the sensitive range of the spectrophotometer. The results from these two groups is therefore not directly comparable. However, the non-I/R group described in 7.4.3 was performed at 1 mM in order to allow a comparison with the groups performed in similar conditions.

The swelling response to this was measured, and the absolute change in absorbance and maximal rate of change of absorbance quantified as end points to the assay. Exemplar traces for P14 and adult hearts are shown in Figure 7-11 and Figure 7-10.

7.3.1 Experimental protocols

Three sets of experiments were performed with these hearts and were all performed in both adult and immature organs. In the first, mitochondria were extracted as described and assessed for calcium induced swelling from hearts perfused for 30 minutes with no further intervention. To this suspension of mitochondria was added cyclosporin A (CsA; in DMSO as a solvent) 2 minutes prior to assessment of swelling (500 nM- a level previously found to completely inhibit pore opening in a similar preparation (Griffiths and Halestrap 1991)). This was to establish this technique as resulting in a viable population of intact mitochondria; with the hypothesis that if the mitochondria were not intact then CsA should not induce any change in their behaviour in the swelling assay.

The second set of experiments investigated the response of mitochondria isolated from hearts not exposed to I/R injury to isoprenaline, a non-selective β adrenoreceptor agonist, whilst the final set of experiments examined the response

following isoprenaline perfusion and a 30- minute global ischaemic injury followed by a 5 minute reperfusion period. At the end of these protocols, the hearts were removed from the aortic cannula, and homogenised as described in Chapter 2. These protocols are shown diagrammatically in Figure 7-1.

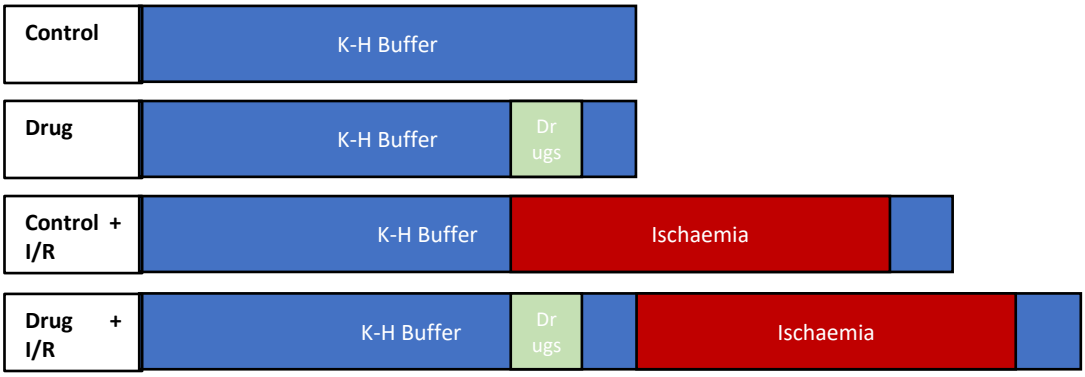


Figure 7-1 Experimental protocols for mitochondrial isolation experiments. Groups indicated in left panel. Colours indicate phases of experiment; green= standard perfusion with KH buffer; orange= perfusion with KH containing a drug; blue= normothermic global ischaemia. Size of blocks proportionate to duration as detailed in text.

7.4 Results

7.4.1 Characterisation of Isolated Mitochondrial preparation

7.4.1.1 The effect of CsA on Mitochondrial Swelling in ex vivo perfused hearts

An initial set of experiments were performed involving hearts taken from adult rats and perfused for 40 minutes with KH buffer. For these experiments, 6 hearts were used to obtain isolated mitochondria. Each of these preparations was then studied for the effect of Ca^{2+}, CsA, and DMSO (used to dissolve CsA) using aliquots of the mitochondria.

Figure 7-2 shows an example family of plots from one of these preparations. At the origin, calcium is added which triggers a swelling response, from which the maximal rate and amplitude may be quantified and compared.

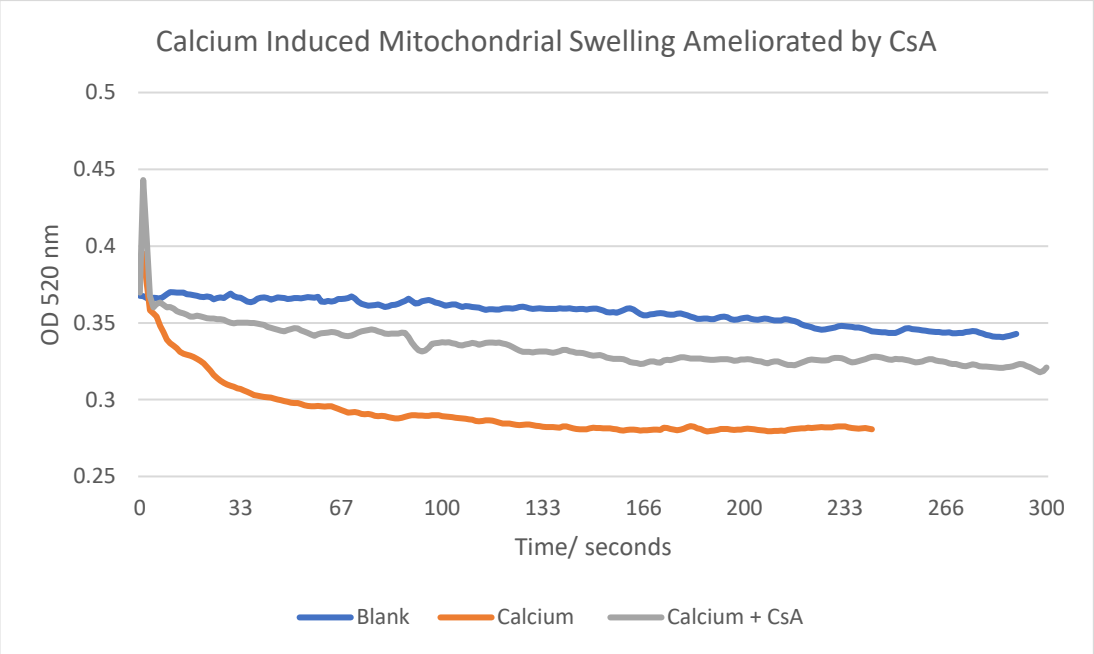


Figure 7-2 Exemplar traces of mitochondrial swelling in preparations from adult heart. Y- axis represents absorption through mitochondrial preparation vs time on x-axis. Change in absorption vs. time represents swelling. Mitochondria pre- treated with or without CsA; the blank group has no further addition. Traces offset to common origin.

Figure 7-3 shows the effects of addition of Ca^{2+} to the mitochondrial isolation preparation from adult hearts. Compared to the change in the untouched baseline trace as a control, the addition of Ca^{2+} to the suspension causes a rapid change in the OD reading, interpreted as representing swelling of the mitochondria; an 8.65- fold increase in the amplitude of swelling over the time period studied.

When CsA was incubated with the suspension for 2 minutes prior to the addition of Ca^{2+}, the amplitude of swelling that resulted was significantly reduced, although it remained greater than that of the baseline trace. DMSO is used as a solvent for CsA; in order to test that the inhibition of swelling was due to CsA alone rather than an action of this solvent, DMSO, which was used as the solvent for both CsA and cAMP analogue solutions, was incubated with the mitochondrial suspension before the addition of Ca^{2+}. This led to a degree of swelling significantly greater than baseline, but not significantly different from that induced by Ca^{2+} alone. This was done in order to demonstrate that subsequent results were not due to the presence of DMSO.

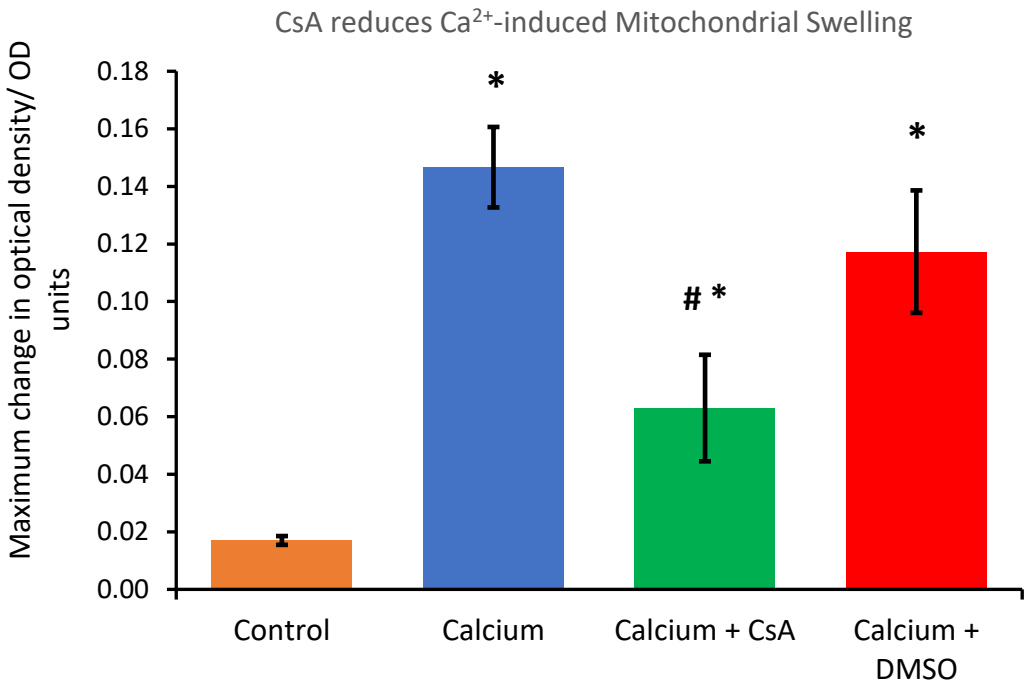


*Figure 7-3 Absolute Change in absorbance of isolated adult mitochondrial preparation after addition of Calcium (1 mM) alone, ± CsA; additional arm with DMSO without CsA. Change in optical density denotes mitochondrial swelling. *= Significant difference from control swelling; #- significant difference to swelling induced by calcium alone. Significance level taken as p<0.05 and performed by Student's T-test; data expressed as mean; error bars represent standard error of the mean. N= 6 hearts per group.*

7.4.2 The Effects of Cardiac β-Adrenoreceptor Stimulation on MPTP Opening in Adult and Immature Hearts not exposed to I/R Injury

The next set of experiments examined the ability of exogenous calcium to induce swelling in the suspended mitochondria, from hearts perfused with the standard buffer (control) or those perfused for 5 minutes with isoprenaline prior to isolation.

These experiments used 6 hearts per age group per drug treatment; i.e.. 12 adults and 12 P14, with 6 in each group given the control treatment or isoprenaline perfusion during the Langendorff stage of the experiment.

Age Group	*Control*	*Isoprenaline Perfusion*
Adult	6	6
P14	6	6

Table 7-1 Numbers of hearts used per age group in experiments comparing pre-ischaemia sensitivity of MPTP with or without prior perfusion with isoprenaline.

For the adult hearts, after pseudo-physiological perfusion i.e. without any ischaemia or reperfusion stimulus, the addition of isoprenaline prior to isolation of mitochondria produced a significant reduction in the stimulated swelling; this was true in considering either absolute amplitude (Figure 7-5) or the maximal rate of change of absorbance (Figure 7-4). For absolute change in swelling, the signal from the isoprenaline treated mitochondria was 26% less than that from the control group; whilst considering rate of change the isoprenaline treated sample showed 59% of the measurement in the control group. Similar significant observations were made in mitochondria derived from P14 hearts (Figure 7-7 & Figure 7-6). Isoprenaline produced a 33% reduction in the absolute magnitude of swelling, and a 40% reduction in maximal rate of swelling.

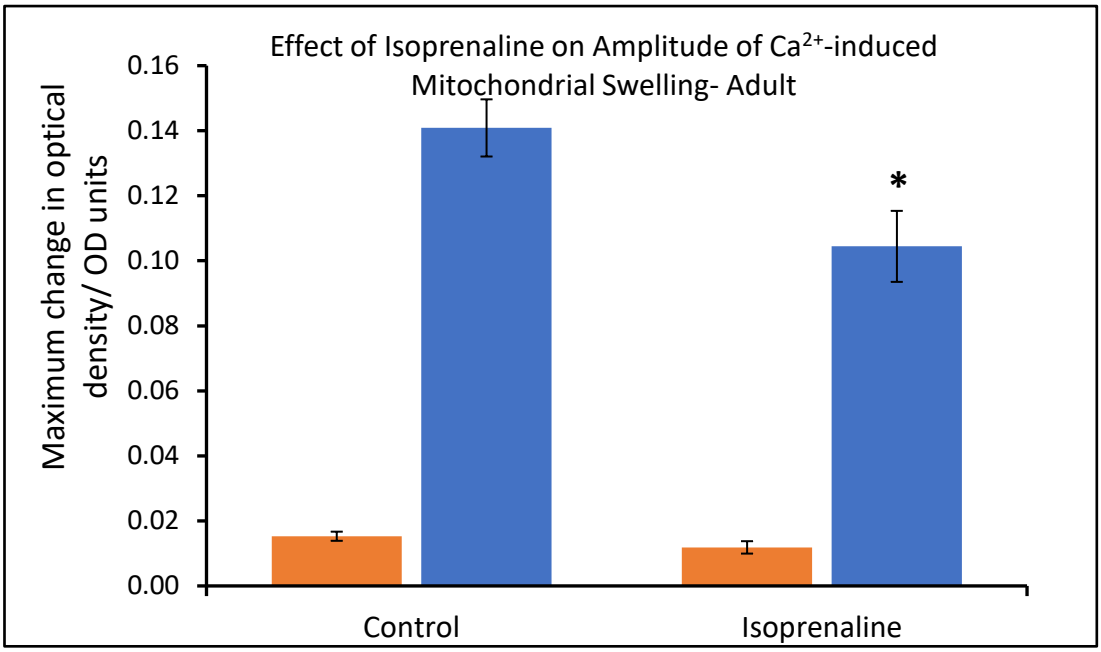


Figure 7-4 Absolute change in mitochondrial swelling following addition of calcium to adult isolated mitochondrial suspension with and without pre-isolation perfusion with isoprenaline measured by absorbance.

*Orange- rate of change in OD during baseline observation; blue- rate of change in OD after calcium addition. *= p<0.05 by Student's T-test; error bars= standard error of the mean. N=6 hearts per group.*

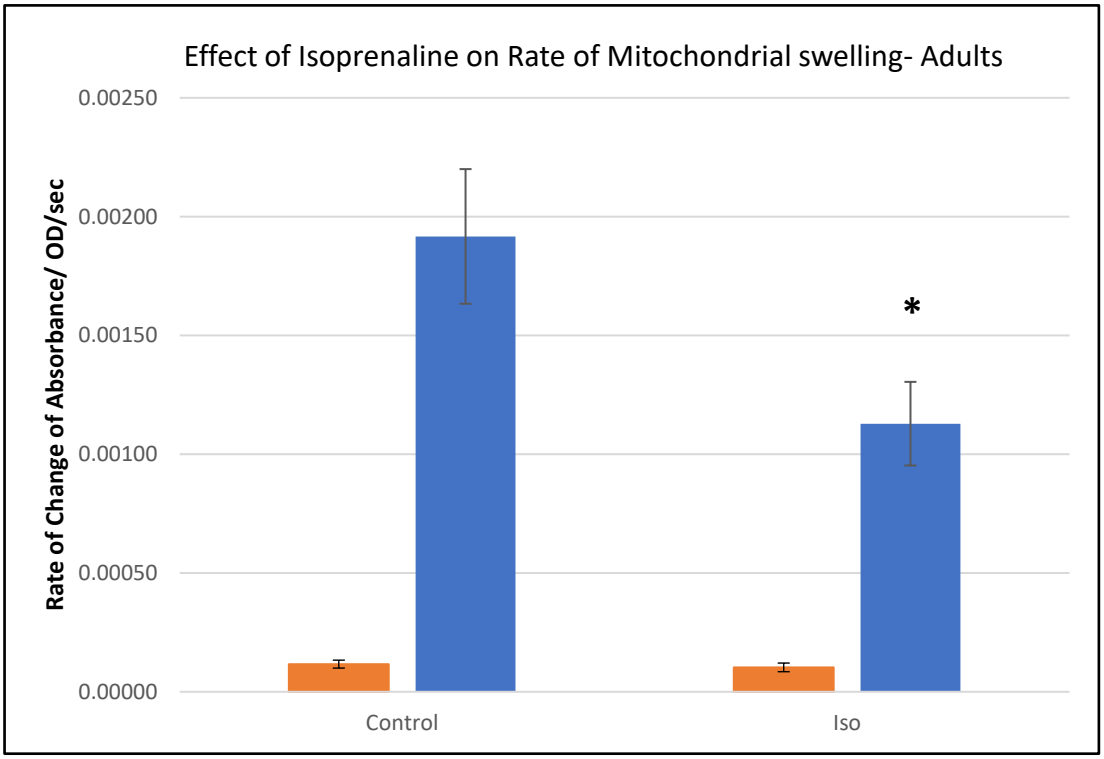


*Figure 7-5 Maximal rate of change in mitochondrial swelling following addition of calcium to adult isolated mitochondrial suspension with or without pre-isolation perfusion with isoprenaline measured by absorbance. Orange- change in OD during baseline observation; blue- change in OD after calcium addition. *= p<0.05 by Student's T-test; data expressed as mean value; error bars= standard error of the mean. N=6 hearts per group.*

The corresponding observations in the P14- derived mitochondria are shown in Figure 7-7 and Figure 7-6. Here, the levels of baseline swelling are much smaller than in the adult at 0.002 vs 0.015 in amplitude, with a similar trend observed for rate of swelling. The amplitude of swelling after induction with Ca^{2+} increases to 0.024 in the control. In the isoprenaline treated group, this falls to 0.018, a significant decrease of 25%. A similar pattern is observed examining the rate of swelling results; with a 29% reduction in the maximum rate in the isoprenaline group vs. control.

All of these observations were statistically significant; so in both adult and P14 mitochondria, isoprenaline significantly desensitised the pore in a model of physiological function.

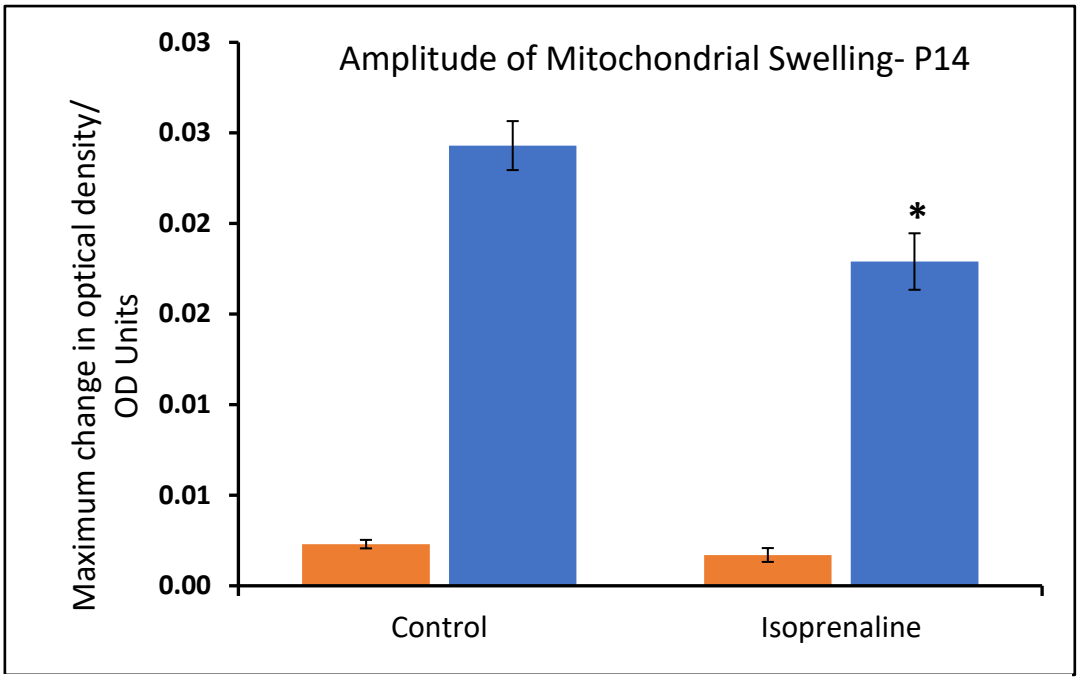


Figure 7-7 Absolute change in mitochondrial swelling following addition of calcium to P14 isolated mitochondrial suspension with and without pre-isolation perfusion with isoprenaline measured by absorbance.

*Orange- rate of change in OD during baseline observation; blue- rate of change in OD after calcium addition. *= p<0.05 by Student's T-test; error bars= standard error of the mean. N=6 hearts per group.*

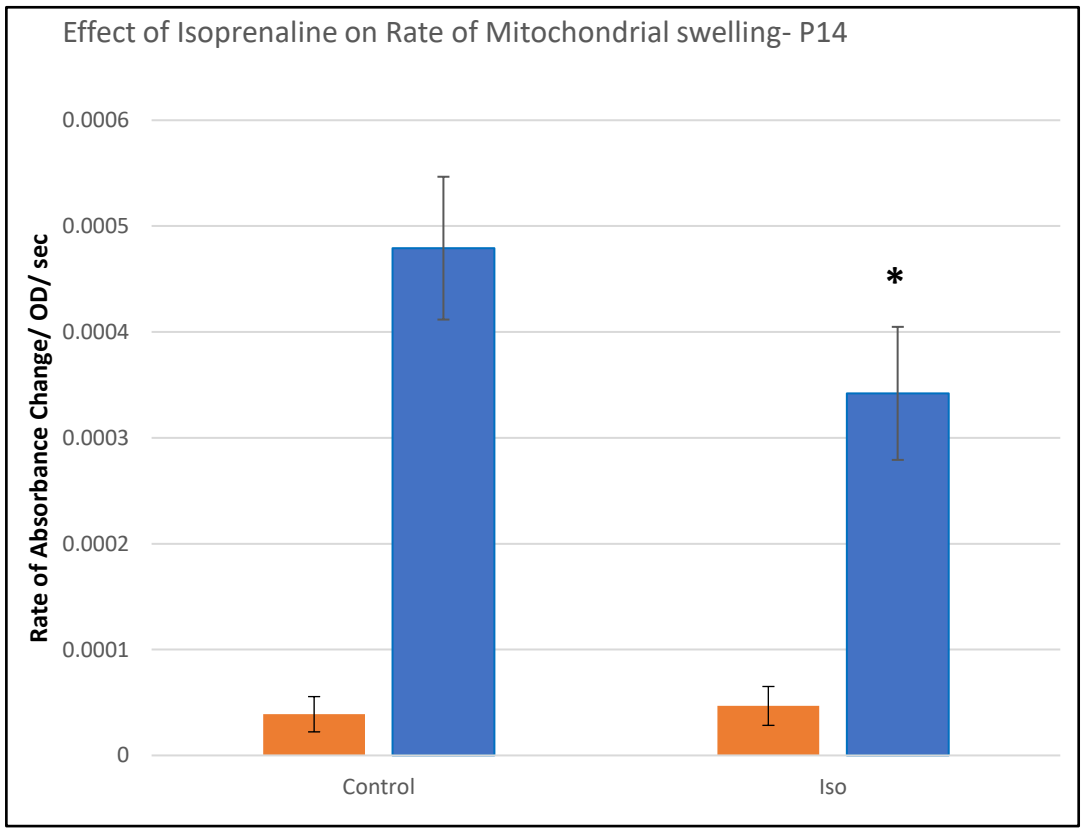


*Figure 7-6 Maximal rate of change in mitochondrial swelling following addition of calcium to adult isolated mitochondrial suspension with or without pre-isolation perfusion with isoprenaline measured by absorbance. Orange- change in OD during baseline observation; blue- change in OD after calcium addition. *= p<0.05 by Student's T-test; data expressed as mean value; error bars= standard error of the mean. N=6 hearts per group.*

7.4.3 The Effect of I/R on MPTP opening

In adult hearts, whether or not exposed to ischaemia & reperfusion, pore opening could be stimulated by the administration of calcium into the mitochondrial suspension. However, the degree of swelling stimulated by calcium almost doubles in hearts exposed to global ischaemia and reperfusion injury prior to isolation of mitochondria. The ability of Ca^{2+} to stimulate opening of the MPTP was shown in Section 7.4.1 with experiments carried out with 3 mM Ca^{2+} added, but demonstrated again in a separate set of experiments at 1 mM for comparability with the pharmacological perfusion data. This effect alongside the other experimental results for this section in **Error! Reference source not found.** and **Error! Reference source not found.**. For all of these experiments involving comparative studies with cAMP analogues and 1 mM Ca^{2+} such as these data, the numbers of animals used and included in the analysis are given in Table 7-2.

Experimental Arm	Adult	14-days
Control (No I/R)	6	4
Control with I/R	5	4
Isoprenaline	5	5
8-Br	5	5
6-Bnz	6	5
CPT	6	5

Table 7-2 Numbers of hearts used for post- reperfusion pharmacological intervention experiments on mitochondria

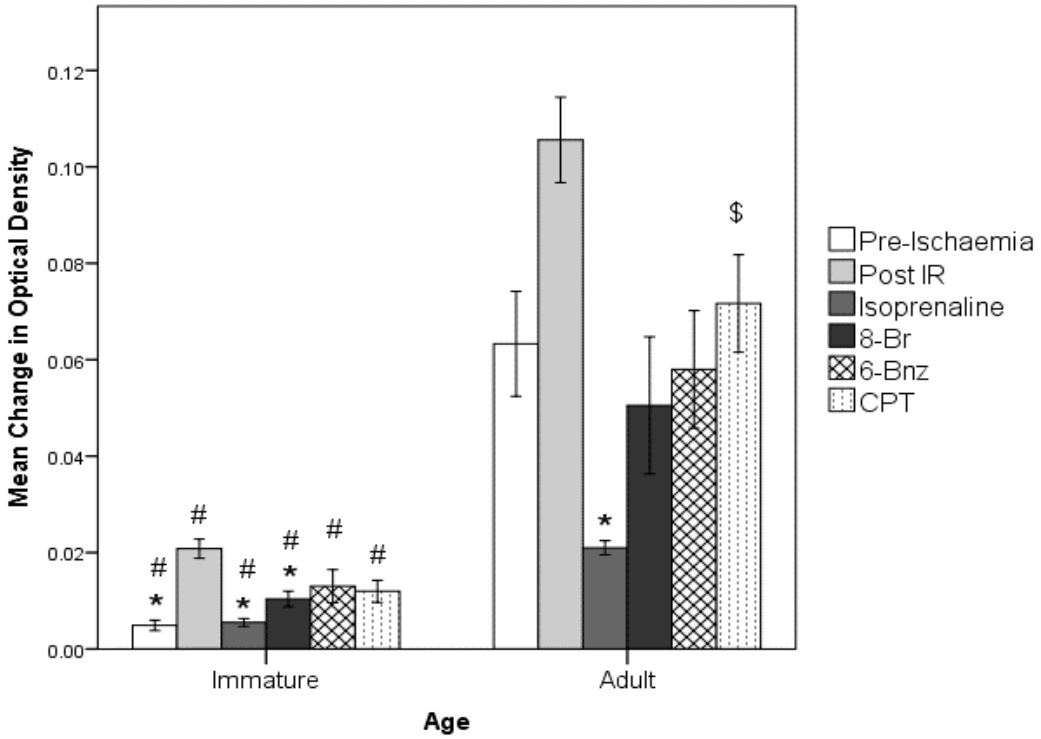


*Figure 7-8 Summary of effects on absolute change in mitochondrial swelling by pre-ischaemic pre-isolation interventions targeting different stages of the β adrenergic signalling pathway on both immature (P14) and adult isolated mitochondrial preparations.Error bars = 1 SEM. *P<0.05, vs IR group in corresponding age group. # P<0.05, vs adult age group in corresponding intervention. $ P <0.05 vs isoprenaline in corresponding age group. Significance testing by Student's T-test. N=6 per group.*

7.4.4 The effect of I/R on MPTP opening with cAMP/PKA/Epac activation

The next set of experiments examined the pore sensitivity in isolated mitochondria from hearts which had been perfused with either isoprenaline or a cAMP analogue prior to a global ischaemic injury and reperfusion. There were two controls in these experiments; mitochondria from hearts not exposed to I/R, and mitochondria exposed to I/R but not perfused with any drugs. In these experiments, as the pore was expected to be relatively sensitive to opening stimuli, the concentration of Ca^{2+} used was 1 mM . They are therefore not directly comparable results to Section 7.4.1, which used 3 mM Ca^{2+}. As discussed in the methods section, the use of 1 mM Ca^{2+} is common in studies of mitochondria following I/R injury (Abellán, Miró-Casas et al. 2006, Khaliulin, Parker et al. 2010). 3 mM Ca^{2+} was used in Section 7.4.1 in order to

increase the magnitude of response in non- I/R injured mitochondria to produce a detectable difference between experimental groups.

7.4.4.1 Effect of Isoprenaline on I/R induced MPTP opening

In adult hearts perfused with isoprenaline prior to I/R, there was a significant reduction in amplitude of swelling (0.0210 ± 0.0015) compared to those hearts receiving no added drug (0.1056 ± 0.0089); this reduction was to below the level of the control hearts not receiving an I/R injury (0.0633 ± 0.0109) and was statistically significant.

A similar pattern at lower magnitude was seen in the equivalent experiments on P14 heart. A significant reduction in amplitude of swelling was seen, falling from a control post I/R result of 0.0208 ± 0.0199 to 0.0055 ± 0.0018 in experiments with isoprenaline. This was comparable to the non-I/R injured control, although not the same supra-normal result, as seen in the adult (0.0049 ± 0.0011). These results are also shown in Figure 7-9 **Error! Reference source not found.**.

A similar pattern was observed in the results for rate of swelling, shown in **Error! Reference source not found.**. For the adult hearts, there was a significant reduction with isoprenaline compared to I/R treated controls (9.75±2.1 x10^{-4} OD/s vs 2.5±0.39 x 10^{-3} OD/s). This approximates to a roughly 2.5 fold reduction in maximal swelling rate, and is comparable to the non-I/R control rate of 7.1±1.4 x 10^{-4} OD/s. All of these differences were statistically significant.

In the immature group, a very significant reduction was observed (4.9±1.7 x 10^{-5} OD/s vs 90.3±18.0 x 10^{-5} OD/s), which was comparable to the non-I/R control swelling rate of 5.1±1.4 x 10^{-5} OD/s.

7.4.4.1.1 Effect of 8-Br

Perfusion with 8-Br, the non-selective agonist of both PKA and EPAC reduces MPTP sensitivity in adult and immature hearts- Figure 7-9. The total amplitude of swelling in adults fell to 0.0505 ± 0.0142 from 0.1056 ± 0.0089 after 8-Br treatment compared to I/R injured controls, and was indistinguishable from the uninjured control (0.0633 ± 0.01099).

In the immature, P14, hearts, there was a clear effect of 8-Br perfusion with the total amplitude of swelling falling from 20.8±1.99 to 10.4±1.6 (X1000) OD Units; however, this remained greater than the level of the non- I/R control.

The data for the change in rate of swelling after 8-Br perfusion are shown in **Error! Reference source not found.**. The overall pattern is similar to that for amplitude of swelling. In the adult hearts, 8-Br perfusion reduces the maximal rate of swelling from 2.5±0.4 x 10^{-3} OD/s to 1.2±0.6 x 10^{-3} OD/s. This is not quite back to the non- I/R control results of 7.1 ±1.4 x 10^{-4} OD/s. In the immature hearts, the maximal rate falls from 9.0±1.8 x 10^{-4} OD/s to 7.7±1.3x 10^{-5} OD/s , again similar to the non- I/R value of 5.1±1.4 x 10^{-5} OD/s.

7.4.4.1.2 Effects of CPT & 6-Bnz

Finally, the effects of CPT and 6-Bnz on amplitude of swelling change and maximal rate of change are also shown in Figure 7-9 and **Error! Reference source not found.**. For the adult group, 6-Bnz and CPT both reduced amplitude of swelling vs the I/R control (6-Bnz, -0.04759 ± 0.01507, CPT, -0.03393 ± 0.01344); however neither of these effects were significant. Likewise, for the P14 group, 6-Bnz and CPT caused non-significant reductions in the total magnitude of swelling.

For the changes in maximal rate of swelling, in the P14 hearts, both CPT and 6-Bnz produced significant reductions in the rate of swelling vs the I/R control (6- Bnz, -7.6±1.0 x 10^{-4} OD/s $p < 0.0005$, CPT, -8.1±0.9 x 10^{-4} OD/s, $p<0.005$) although these remained notably greater than the non-I/R control (5.1±1.4 x 10^{-5} OD/s). In the adult hearts, whilst both CPT and 6- Bnz produced observable reductions in swelling rate, only 6-Bnz reached significance; 1.2±0.4 x 10^{-3} OD/s, $p = 0.041$.

7.4.5 Summary of Interventions

Administration of drugs acting along the β-AR linked signalling pathway prior to ischaemia-reperfusion can ameliorate sensitization of permeability transition following ischaemia/ reperfusion injury. Figure 7-11 and Figure 7-10 show exemplar traces for each drug/ condition in the adult and immature heart demonstrating graphically the changes in amplitude of mitochondrial swelling that occur after addition of Ca^{2+}. The overall effects of the experiments are summarised in **Error! Reference source not found.**Figure 7-9 and Error! Reference source not found.**.**

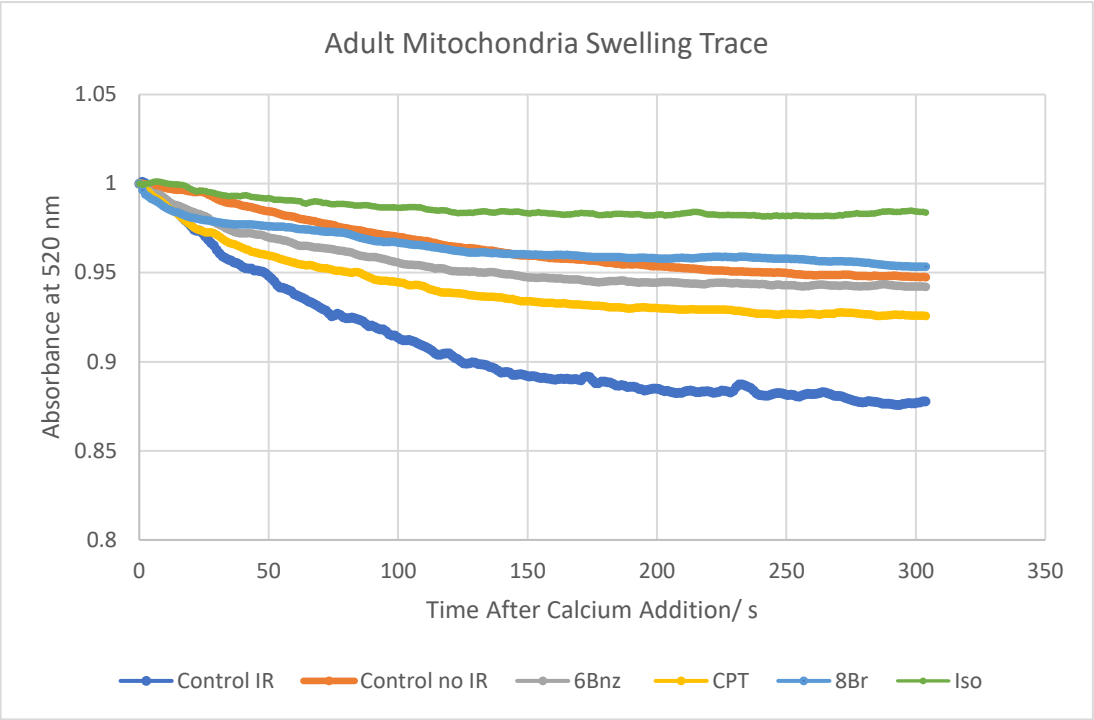


Figure 7-10 Exemplar traces of mitochondrial swelling vs time (s) in isolated mitochondrial preparations from adult heart, pre- treated with or without IRI and drugs. Traces offset to common origin.

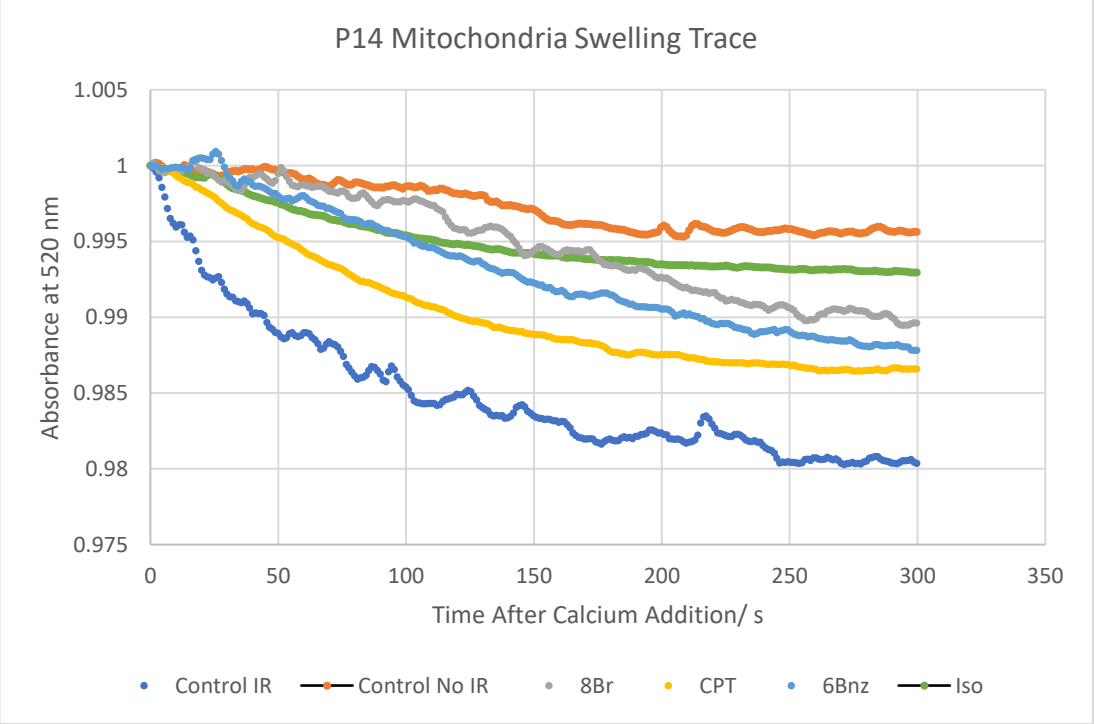


Figure 7-9. Exemplar traces of mitochondrial swelling vs time (s) in isolated mitochondrial preparations from 14 day old heart, pre- treated with or without IRI and drugs. Traces offset to common origin.

In the adult, this effect is most marked with isoprenaline which comes closest to a complete rescue from the sensitization from ischaemia/ reperfusion injury; and to a lesser extent with the non-selective cAMP analogue 8-Br. The effect is also present but to an even smaller degree with either CPT, or 6-Bnz, the selective agonists for EPAC and PKA respectively.

When assessing the peak swelling rate of mitochondria after calcium stimulation (**Error! Reference source not found.**) a similar but not identical pattern was observed. Swelling could be stimulated without any deliberate injury to the mitochondria; from hearts perfused without any ischaemia/ reperfusion injury. Those hearts exposed to I/R alone without any further injury swelled with the greatest speed, whilst isoprenaline pre-treatment caused maximal reduction in the rate of swelling. 8-Br also reduced rate of change to an indistinguishable degree as isoprenaline; in this measurement 6- Bnz had a similar effect to 8- Br, whilst CPT caused a reduction in the rate of swelling but only to a lesser degree than the other agents.

In the experiments on immature, P14 hearts, swelling was observed following exposure of mitochondria extracted Langendorff perfused hearts to calcium. In the group not exposed to I/R, the amplitude and rate of this swelling was minimal. Treatment of these hearts with the 30 minute I/R injury but no other intervention produced maximal amplitude and rate of swelling. Isoprenaline was the most effective intervention at rescuing the mitochondria from calcium- induced swelling following I/R injury; this treatment reduced swelling down to the level of the hearts not exposed to I/R injury. 8-Br produced a similar reduction, but in the rate of swelling only. The net amplitude of change was reduced with 8-Br, but not to the level of Isoprenaline; it was however significantly different to the reduction in amplitude seen with either 6-Bnz or CPT. CPT showed a non-significantly different reduction in rate of swelling; however, 6-Bnz showed less of an ability to reduce

swelling rate than 8-Br, although it still produced a degree of amelioration vs. the I/R control.

Therefore, considered relative to the adult experiments, the overall pattern was maintained, but for each arm, the amplitudes and rates of swelling were reduced in magnitude compared to the adult equivalent. The most effective agents were those that caused activity of both PKA and EPAC; that is, isoprenaline and 8- Br. The individual agonists produced intermediate results; and in the adult, 6-Bnz seemed to ameliorate swelling more potently than CPT, although the experiments were not designed to consider such a difference.

7.5 Discussion

Key findings:

1- Isolated mitochondria from both adult and 14-day swell up in response to Ca^{2+}
2- Mitochondrial swelling can be reversed by CsA confirming MPTP opening
3- MPTP sensitivity to Ca^{2+} is lower in 14-day mitochondria compared to adult
4- I/R increases sensitivity of MPTP to Ca^{2+} in both age groups which is fully reversed in hearts transiently pre-treated with Iso
5- Pre-treatment with 8-Br induces similar response to Iso whilst pre-treatment with CPT & 6Bnz reverse the effect but to a lesser extent compared to 8Br

7.5.1 Isolated mitochondria are intact and their Ca^{2+}-induced swelling can inhibited by CsA indicating MPTP opening

Whilst this method for isolation of mitochondria has been used by several research groups with success, it was a new technique in our group. Therefore, in order to validate this method, a series of experiments incubating CsA with the mitochondrial suspension were performed.

The demonstration that addition of CsA to the suspension inhibited mitochondrial swelling in the presence of calcium, shows that that swelling occurs by induced opening of the MPTP. CsA is known to be a potent inhibitor of the pore through interaction with cyclophilin D (Elrod, Wong et al. 2010) which is its only known means of interaction with the mitochondrial pore, at a dose range similar to that used in this experiment (Griffiths and Halestrap 1991). Therefore, the observation that incubation of this suspension with CsA is able to ameliorate Ca^{2+} induced swelling implies that the mitochondria isolated in this way are intact with a functional pore arrangement. This serves to validate this preparation for the experiments which follow.

7.5.2 The Immature Heart's Mitochondria are Less Susceptible to Ca^{2+} Induced Swelling via the MPTP than the Adult Heart's

When the control experiments (those hearts not perfused with any drugs added to the buffer without 30 minutes of ischaemia & 5 minutes reperfusion) are considered, I have shown that there is a measurable degree of swelling of the mitochondria in response to calcium in both adult and immature hearts. This degree of physiological sensitivity is demonstrably and significantly greater in the adult heart as compared to the immature; so whilst it is possible that this phenomenon may reflect unintended injury through the perfusion and isolation process, that different groups exhibit this observation to differing degrees implies there is a physiological basis to this feature. The proteomic data discussed in Chapter 3 demonstrates a divergence in the abundance of many proteins involved in mitochondrial regulation and function, including some of the postulated components of the MPTP. It is possible that this difference in expression contributes to the observed differences, both in this physiological model and in the I/R experiments. Further, the MPTP itself is even more poorly characterised in the immature heart in terms of its components and structure than in the adult; it may be that differences in these characteristics could account for the observed differences.

The overall physiological role of the MPTP in contexts aside from ischaemia and reperfusion injury remains unclear, and further studies on the MPTP in its intracellular environment is needed to address this.

7.5.3 Ischaemia & Reperfusion Injury Sensitises the MPTP to Ca^{2+} in both Immature and Adult Hearts

Both age groups demonstrated a significantly larger swelling response to calcium following exposure to I/R injury. The MPTP is well described as being sensitized to opening stimuli following ischaemia and reperfusion injury, which is thought to be as a consequence of the influence of reactive oxygen species. Therefore, these observations are expected in the context of prior work (Suleiman, Halestrap et al. 2001), but do confirm this work as a valid model of the known effects of reperfusion injury.

7.5.4 The MPTP is less likely to open in the Immature Heart exposed to Injury than the Adult Heart

Although the proportionate change in sensitivity to MPTP opening after I/R is greater in the immature control hearts, the absolute magnitude of this swelling response is less than a fifth that seen in the adult heart. A similar pattern is seen concerning mean rates of swelling. This is strikingly similar to the vulnerability pattern to whole heart global ischaemia and reperfusion injury, described as 'bell-shaped' or biphasic wherein vulnerability is thought to decrease from birth to 2 weeks post-natal age in the rat, before rising again to a peak at adulthood (Riva and Hearse 1993, Ostadalova, Ostadal et al. 1998). It is therefore appealing to suggest that at least one of the factors underpinning this change in vulnerability, is changing behaviour of the MPTP with age (Balaska, Halestrap et al. 2005). Why the MPTP should change with age is not clear; the structure of this pore is still a matter of debate, and its constituents are still not comprehensively clear (Ong, Samangouei et al. 2015). The structural knowledge that does exist is from work in adult tissues, and so cannot be assumed to remain consistent in the immature heart. It is further possible that mitochondrial morphology differs in the immature heart to that of the adult, which may have further consequences for the vulnerability to swelling.

7.5.5 The MPTP may be induced to open under physiological conditions and this may be ameliorated by Isoprenaline

The role of the MPTP during physiological conditions is still a matter of debate, and so the observation here that firstly opening in uninjured samples can be induced, but more so that that opening can be desensitized by isoprenaline was moderately surprising and if confirmed is a significant finding.

The most common view is that of the physiological role for the pore is that of 'flickering' or short opening (Hausenloy, Wynne et al. 2004, Wang, Fang et al. 2008), in which brief bursts of opening contribute to maintenance of mitochondrial and cytoplasmic Ca^{2+} levels. This view is questioned- De Marchi, for instance (De Marchi,

Bonora et al. 2014) not finding a role for the MPTP in the maintenance of Ca^{2+} homeostasis in cultured cells.

The evidence shown here, however, shows that the MPTP in this preparation may be induced to open in a suspension of isolated organelles from hearts not exposed to any injurious stimulus; at least, presuming that the process of isolation is not in itself a similar stimulus to ischaemia and reperfusion of the heart. This would support a role for the MPTP changing permeability dynamically in the usual function of the heart. This opening of the pore seems to differ between adult and immature hearts; the P14 mitochondrial extract being significantly less sensitive to induction of permeability transition than the adult. This is similar to the pathophysiological changes seen after I/R described in Section 7.5.2.

Further, these experiments also show that non- I/R induced swelling may be significantly ameliorated by isoprenaline in both the adult and immature heart. Isoprenaline, whilst not a physiologically occurring substance in the heart is a non-selective β adrenoreceptor agonist and therefore pharmacologically functions in some respects similarly to adrenaline. Speculatively then, it might seem reasonable that under situations of physiologically increased oxidative stress, such as a sudden need to increase ATP production in response to demand, concurrent release of adrenaline as part of the physiological sympathetically mediated stress response serves to inhibit MPTP opening and reduce damage incurred by this increased ROS flux. The MPTP is also thought to be part of the synthetic machinery for ATP; it may be that these observed changes in response to adrenergic stimulation help to facilitate a response to increase ATP demands.

7.5.6 Signalling along the β- Adrenergic Receptor linked pathways can desensitize the MPTP to opening following I/R injury

7.5.6.1 Protection at the Receptor Level

Pre-treatment with isoprenaline reduces the sensitivity of isolated mitochondrial swelling to calcium and brings it to the level of the non-injured experiments. Isoprenaline is a non-selective agonist at all isoforms of the β- adrenoreceptor (Leone, Albanese et al. 2002). The primary mode of action of isoprenaline at these

receptors is thought to be by indirect activation of adenylate cyclase, whose action is to catalyse the intracellular formation of cAMP (Woo and Xiao 2012). This, however, is not the only mode of action of isoprenaline at this receptor; a secondary function may be to catalyse the formation of inositol triphosphate as another second messaging system (Endoh 1995). It does seem as though isoprenaline therefore indirectly modulates pore opening in both age groups studied. Combined with the observation that pore opening occurs in physiological conditions, this could mean that sympathetic activity has a role in modulating mitochondrial function in normal circumstances. This work however, does not show mechanistically how isoprenaline may be mediating these effects. I have shown previously (Lewis, Szobi et al. 2018) that isoprenaline linked whole-heart cardioprotection is correlated to glycogen depletion, and others have shown (Halestrap and Richardson 2015) a link to dissociation of hexokinase from the mitochondrion. A study linking these features together may well explain the observations of the properties of isoprenaline seen here.

7.5.6.2 Protection is Replicated by cAMP Analogues but requires PKA & EPAC synergy

Prior perfusion of hearts with 8-Br reduces the sensitivity to swelling of both adult and immature mitochondria to almost, but not quite, the level seen with isoprenaline. This study was not powered or intended to be seen as a non-inferiority study between these two compounds, however. It may be that isoprenaline acting at the receptor level stimulates other occult pathways of signalling in parallel, or that there is a different amplitude of response along the downstream communication channels.

This study has however demonstrated that at a mitochondrial level, a cAMP analogue stimulating both PKA and EPAC produces protection against ischaemia and reperfusion injury. This effect is not seen in either age group following stimulation with either CPT or 6-Bnz alone, where the response is not significantly different to that following I/R alone. This may imply that the reduction in sensitivity is due to a synergistic effect of the action of PKA and EPAC. It is, however, also possible that the protection depends on a mechanism not stimulated by either of these agents but

which is by an off-target effect of 8-Br. It would be interesting to examine the effects of perfusion with both CPT and 6-Bnz prior to I/R and mitochondrial isolation, to see if the effects of 8-Br are replicated. A further possibility would be to use the commercially available inhibitors of PKA and EPAC, alongside perfusion with 8-Br, in order to independently confirm the requirement for synergy.

8 Summary, Conclusions, Limitations & Further work

8.1 Summary of Findings & Overall Discussion

The results described here have shown physiological and pathological distinctions between the adult and developing heart in a number of domains which change as the heart matures.

The proteomic findings, which inform the remainder, have shown that there are molecular changes which track with previously known and experimentally observed patterns of changes in vulnerability of the developing heart to ischaemia & reperfusion injury, including in networks involved in Ca^{2+} homeostasis, regulation of mitochondrial dynamics and function, antioxidant activity, and signalling involving cAMP. This confirmed the hypothesis for this work at the outset, that cAMP signalling was likely to be of consequence to cardioprotection of the developing heart, and that differences observed in the vulnerabilities between adult and developing hearts may be accounted for through these changes.

Interventions which were previously demonstrated to be cardioprotective in the adult heart were then also demonstrated in the developing heart. Whilst in the adult, consecutive perfusion with isoprenaline and adenosine was previously known to be protective, a finding replicated here, in experiments using an equivalent ischaemic time the developing heart did not appear to share this effect. This may have been because the intrinsic vulnerability of these hearts was quite low, and so a protective signal could not be seen against this background. However, when 8-Br, the non-selective agonist of PKA & Epac, was studied in similar experiments, it protected the adult heart to a similar degree as the previous isoprenaline/ adenosine study. Again, with a matched ischaemic time of 30 minutes, the developing hearts did not show a protective effect of 8-Br. However, an additional group with an ischaemic time of 50 minutes was added to the P14 age group. This group showed an equivalent histological degree of injury to the adult controls exposed to global ischaemia for 30 minutes. When the 50 minute P14 group was perfused with 8-Br prior to ischaemia, a protective effect was then seen to a biologically significant degree. This effect was not seen with perfusion with either 6-Bnz or CPT, the PKA and Epac selective agents

respectively, suggesting synchronous activity of both intermediaries is necessary for the protective effect, perhaps through synergism.

This group of interventions was then studied at the level of the isolated cardiomyocyte using H_2O_2 and elevated Ca^{2+} concentrations to simulate the environment of a cardiomyocyte during reperfusion. Here, the first group of experiments sought to demonstrate the central role of the Mitochondrial Permeability Transition Pore in cell death during reperfusion. Incubation of cyclosporin A with adult cardiomyocytes significantly ameliorated the rate of cell death in the simulated reperfusion conditions. However, at P28 this effect was only seen to an intermediate degree, and in the P14 group was entirely absent. This may reflect an underlying difference in the regulation or composition of the MPTP in the developing heart; however, this group had a very high intrinsic death rate so the observation may be due to damage incurred through the process of isolation. When these cardiomyocytes were incubated with PKA and Epac agonists, the rate of death in simulated reperfusion was ameliorated with simultaneous activation of PKA & Epac, although selective activation of either was not sufficient to protect the cardiomyocytes against death. Examination of the mechanism of EC coupling in these cardiomyocytes perfused with the Epac and PKA agonists showed that when exposed to cyanide in a model of ischaemic injury, the synergistic activity of both caused preservation of contractile function and Ca^{2+} transients in both adult and P14 derived cells, as well as a reduction in death rate similar to that seen on exposure to H_2O_2. However, this was associated with an increased propensity to arrhythmia of the cardiomyocytes.

The mitochondrial studies following on from this work then gave some insight into the mechanisms of this protection. It demonstrated that in a model of I/R injury, mitochondria derived from P14 hearts are intrinsically less likely to undergo permeability transition in response to elevated Ca^{2+} levels. This observation may at least in part account for the observed resistance to I/R injury of the developing heart. When these hearts underwent perfusion with either isoprenaline alone, or Epac and PKA agonists prior to I/R injury and mitochondrial isolation, the isolates from both age groups showed a reduced propensity for MPTP opening when stimulated with

Ca^{2+}, thus suggesting a potential end effector for this cardioprotective intervention. However, this study did not examine the intermediate steps and so cannot definitively demonstrate mechanism. Another interesting observation was that in both adult and P14 mitochondria from hearts not exposed to I/R injury, isoprenaline could also reduce MPTP Ca^{2+} induced injury. This hints at a physiological role for the MPTP under conditions of high sympathetic flux.

In general, this work has shown that in both adult, and immature hearts, stimulation of both PKA and Epac prior to ischaemia & reperfusion injury is cardioprotective. These findings were replicated across whole perfused hearts as well as individual cardiomyocytes and so are relatively robust. Further, it has demonstrated that immature hearts are more resistant to injury than adult hearts, and that that resistance may be due to differences in the MPTP response to oxidative stress.

Recent work has demonstrated a cardioprotective role for PKA & Epac stimulation prior to ischaemia & reperfusion injury (Khaliulin, Bond et al. 2017). However, the observation that this protection also extends to developing hearts is a new and previously undescribed finding.

It has been previously shown that stimulation of the cardiac β-adrenoreceptor with noradrenaline or isoprenaline, classically thought to act through PKA, triggers cardioprotection in a manner much like ischaemic preconditioning (Asimakis, Inners-McBride et al. 1994, Lochner, Genade et al. 1999). Thus the findings here that stimulation with either a PKA or Epac agonist provides a partial protection against injury in measures of cardiac function, biochemical injury and cardiomyocyte survival is consistent with what is understood (Duquesnes, Derangeon et al. 2010). The observation that protection is dependent on the combined activity of both PKA and Epac has recently come to be accepted; indeed others have demonstrated through the use of the PKA and Epac inhibitors H-89 and ESI-09 (respectively) in combination with simultaneous PKA/Epac activity that the cardioprotective effect given by 8-Br perfusion is reduced. Others have studied alternative means of activating PKA and Epac; Mangmool & colleagues (Mangmool, Hemplueksa et al. 2015) examined isolated cardiomyocytes and found an Epac and PKA- dependent cardioprotective effect on stimulation of the GLP-1 (Glucagon- like peptide- 1) receptor with the

agonist exendin- 4. Interestingly, this study was an early demonstration of the cardioprotective effect of synchronous PKA and Epac stimulation in developing cardiomyocytes, also. It is therefore thought that Epac activation is a necessary step to maximal cardioprotection through targets or mechanisms not shared in common with PKA.

Epac is known to activate phospholipase C amongst its targets (Cazorla, Lucas et al. 2009), which in turn leads to PKC activation and IP_3 linked Ca^{2+} release. In particular, it has recently been shown that Epac activation via the β- adrenoreceptor leads to an isoform specific activation of PKCε (Li, Cai et al. 2015). This form of PKC is thought to be involved in cardioprotection, and so this is a potential mechanistic explanation for the observations in this thesis.

The observations of protection in the immature heart are novel and not previously described elsewhere. The first component of this is the reconfirmation of previous observations that the developing heart is less susceptible to I/R injury than the adult; Chapter 4 demonstrated that 50 minutes of global ischaemia was required for a biochemically and histologically identical injury to that shown after 30 minutes in adult rat hearts. Further, Chapter 7 demonstrated a qualitatively similar pattern in the opening sensitivities of the MPTP in mitochondria derived from these hearts. This is a similar finding to the literature for I/R resistance in immaturity (Ostadalova, Ostadal et al. 1998, Ostadal, Ostadalova et al. 2009, Ostadal, Ostadalova et al. 2014), which is thought to be due to developmental changes in cardiac energetics and mitochondrial function (Modi and Suleiman 2004). This is also matched with clinical findings of developmental variation in vulnerability (Imura, Caputo et al. 2001) recognised amongst the surgical community.

The second component to this assertion of protection in the immature heart is the demonstration of protection at the levels of whole heart, myocyte, and mitochondria. The key observation found is that I/R injury does cause increased sensitisation of the MPTP and that that sensitisation may be mediated by PKA & Epac activity.

The mitochondria in the developing heart undergo significant changes during ontogeny. There is a significant increase in number and total volume (Anversa, Olivetti et al. 1980), as well as in their subcellular localisation. There are significant differences in the mitochondrial membrane potential compared to the adult as well; newborns exhibit a single population of mitochondria with a relatively high membrane potential. A switch occurs at the weaning period- the 14 day post-natal stage examined here- where a population with a significantly lower membrane potential appears (Ostadal, Ostadalova et al. 2009) which seems to be related to the intracellular location. The reasons why this may relate to resistance are not entirely clear, but it is attractive due to the linkage between the fall in membrane potential with MPTP opening leading to mitochondrial rupture and cell death (Bernardi, Scorrano et al. 1999, Bernardi 2013).

The results of the experiments with MPTP inhibitors on isolated cardiomyocytes were also significant. CsA did not provide protection against reperfusion- induced cell death in the immature heart as it did in the adult. This is similar to prior reports in similar experiments with SfA, another pore inhibitor (Ostadal, Ostadalova et al. 2009). This then leads the the inference that immature cardiomyocytes may have a lower level of mitochondrial cyclophilin D, the MPTP component which acts as the receptor for CsA. The reasons for this, or other suppositions about the lack of protection, are not clear due to the lack of direct knowledge about the presence, density, composition, and function of the MPTP in the immature heart.

Nevertheless, the observation that the immature MPTP is less sensitive to Ca^{2+} induced opening, and even less so with exogenous signalling through PKA & Epac, strongly supports the view that the mitochondria are at the very least a strong component of the relative resistance of the developing heart to I/R injury, and that this is a modifiable resistance. This suggests an avenue for further investigation into the role of the MPTP in the developing heart, and pharmacological avenues for recapitulating that resistance therapeutically.

8.2 Limitations

8.2.1 Limitations with *ex vivo* perfusion experiments

Whilst there are a number of caveats and drawbacks to the use of *ex vivo* perfusion in research, the Langendorff method is well established and these are well understood (Bell, Mocanu et al. 2011).

Within my experimentation using the Langendorff method, a significant degree of variability was observed with these experiments, such that relatively large variation from experiment to experiment was seen. This limits the size of effect that can be confidently identified. There are a number of reasons that might account for this variation. *Ex vivo* organ perfusion is first subject to variability in the organs taken from animals. The rats used were all of the same breed and sex, and sourced from the same organisation over a limited time period. These measures seem to be as much as can be easily done to limit this variation, short of increasing the numbers of animals used significantly which expands the scope of the study.

Langendorff perfusion also requires rapid cannulation of the aorta in order to re-establish coronary perfusion. This is more straightforward in the larger, adult hearts, becoming more technically challenging as the hearts decrease in size. Although all the hearts included in these results were cannulated within a defined period of time, the neonatal hearts were more likely to be discarded and so although the times to perfusion were not recorded beyond meeting or not the threshold, it is probable that some would have required multiple attempts at cannulation or a prolonged time to perfusion. There is a significant leaning curve and technical difficult to the study of small vessels and organs, and so this limited the number of immature hearts that could be studied and also presents difficulty in the study of younger hearts. Ideally, a 7- day group or even younger would have been included in the experiments involving Langendorff perfusion, but this would have increased the failure rate and time taken to perform the study.

There are also multiple points of technical variation during an experiment. The introduction of an intraventricular pressure sensitive balloon is a significant source of error; the balloon positions itself variably inside the LV cavity, and the diastolic

pressure that resulted from its placement was not controlled between experiments. Significant changes in developed pressure occurred with stepwise inflation of the balloon; this likely reflects changes in the LVEDP. These effects were particularly evident in the 14- day group with smallest balloons requiring very small inflation volumes. The transducers are also subject to electrical interference which could not be eliminated limiting the pressure resolution of the balloon/ transducer system which also limited the accuracy of the functional measurements.

The experimental design of the isoprenaline/ adenosine experiments also did not contain a P14 group with an ischaemic injury matched to the size of the adult injury, which was included in the later experiments with cAMP analogues. This may account for why no protective effect was seen, analogous to the effects seen with the cAMP analogue experiments where a time- matched injury did not produce a protective effect but a magnitude- matched injury did show that effect.

8.2.2 Limitations with proteomic experiments

There are multiple points of inaccuracy that can be considered in the proteomic analysis described in Chapter 3. Intrinsic problems of the method, such as the problems of false discovery, missing values and external validity, are widely discussed in the specialist literature. However, there were limitations specific to the experimental approach chosen here.

The extraction method used was homogenisation with beads in RIPA buffer. Other groups have used other extraction methods in the proteomic literature, with use of a 4M Urea buffer. However, these buffers have mainly featured in the cancer literature with a particular interest in extracellular matrix proteins and seem less suitable for analysis of membrane- bound, cytosolic or nuclear proteins than RIPA buffer and so we elected to use RIPA buffer whilst being mindful of the potential selection bias that this introduces.

Indeed, as an example of this limitation, the proteomic experiments identified over 9000 unique proteins across all of the experimental runs and age groups. This would, at first glance, appear to offer reasonably comprehensive coverage of the myocardial proteome. However, not all of these proteins were found in every group or

experiment. Only 47% of proteins were in common to all. This is not likely to be because the remainder were not found in a particular group. The analysis of the MS/MS data involves assigning a confidence score to each peptide/ protein; conventionally those that are not identified with high confidence are discarded. It is likely that the majority of proteins missing from each sample were in fact represented, but did not appear in the final analysis due to exclusion of lower confidence results.

The use of proteomic TMT-MS/MS techniques for quantification of relative protein abundance, especially of individual proteins, is also not as well validated as other methods such as Western blotting. This lack of validity would best be remedied by using these alternative methods to confirm the results. Also, the use of a standardising reference protein in order to normalise these results would be ideal- GAPDH is often used for this purpose and would further validate the findings. However, this approach is not straightforward in comparative studies of multiple age groups. Housekeeping proteins such as GAPDH used for normalisation are known to vary across ages; therefore finding a suitable reference protein with an unchanging abundance throughout is not trivial; this is problematic in the experimental design chosen here to find meaningful comparisons across age groups.

In addition, these experiments were carried out in male Wistar rats. It is possible that there is intersex variation in protein expression at different ages- significant differences in comparative vulnerability to I/R between males and females at a given developmental stage have previously been noted (Ostadalova, Ostadal et al. 1998). The wider validity of these results is also questionable; much of the experience of the resistance of the heart to injury comes from the human and clinical experience, and indeed the ultimate therapeutic target dependent on this knowledge would be in the human. It cannot, however, be assumed that the proteomic changes observed here would map identically to human developmental milestones.

8.2.3 Limitations with Cardiomyocytes Superfusion Experiments

A clear limitation of this study in approaching the hypothesis on the protective effect of CsA was in the methodology used to isolate cardiomyocytes. Consistent with earlier findings from this Lab, the rate of spontaneous death of cardiomyocytes isolated from immature cardiomyocytes was higher than adults. This could be due to the degree of injury sustained by immature cardiomyocytes in the process of isolation.

There are many steps through the process of isolation at which an age- varying injury may be introduced. The quality of perfusion was not assessed during isolation; in whole- heart studies I have previously assessed functional and biochemical measures (creatine kinase release, contractility, estimated coronary vascular resistance) as measures of the quality of ex vivo perfusion. Those measures are not available for this technique, and so the stringent exclusion criteria I have previously applied for Langendorff perfusion could not be applied.

Another point of variation during isolation is in the process of cardiomyocyte digestion the end point is subjective- essentially judged by tactile assessment, and is therefore very subject to heterogeneity. As the feel of a very small immature heart is clearly different to that of an adult, there is the potential for a systematic bias in the degree of digestion to be introduced. Further, a key obstacle in the development of attempts to freshly isolate cardiomyocytes was in the control of calcium concentration during isolation. This was achieved through the use of chelating agents and slow, stepwise reintroduction of Ca^{2+}; however this was an entirely empirical process based experimentally on what produced a reasonable yield of viable cardiomyocytes. Using the same method for both adults and developing hearts in ignorance of the changes in their calcium homeostasis may produce a non-quantified independent variable.

These experiments also used externally applied hydrogen peroxide and calcium as a means of simulating reperfusion injury. Whilst hydrogen peroxide is well understood to be one of the main ROS species involved in I/R injury, in the intact heart that will be produced in the region of the mitochondrion. In this model, it is applied exogenously, and so must diffuse across the cell membranes and cytosol to reach the

mitochondrion. Thus it is possible that the degree of injury to non-mitochondrial structures differs between a whole heart model of I/R injury or indeed the original pathophysiological description, and the model I use here in studying isolated cardiomyocytes.

Further, the experiments described here do not directly study the MPTP. It is inferred that death is occurring by mitochondrial swelling caused by pore opening and that CsA is exerting an action to inhibit death by pore inhibition, but this has not been definitively demonstrated by this set of experiments. However, a comparison between this method and another more direct way of studying the MPTP, the [^{3}H]2-deoxyglucose ([^{3}H]2-DG) entrapment technique, shows that they produce similar results (Griffiths and Halestrap 1995, Wong, Steenbergen et al. 2012)

8.2.4 Limitations with Contractility and Calcium Transient Experiments

A significant drawback with this study has been the use of cyanide as a metabolic inhibitor. In the context of the wider investigation, the intention is to simulate ischaemic conditions. However, cyanide is a complex IV inhibitor, and so functions by inhibition of oxygen utilisation; it therefore produces a form of hypoxia. While this is similar, an ischaemic insult differs in many ways and it is difficult to speculate if any of those differences are relevant to this study. Nonetheless, cyanide is widely used in similar preparations by many other groups for this purpose, and in many respects produces similar consequences; acidaemia, inhibition of ATP production, and calcium overload.

As for all experiments I have carried out with the cAMP analogues, they should not be considered to be perfectly targeted agonists of the intermediaries PKA and EPAC (Christensen, Selheim et al. 2003). Others have described significant off-target interactions; some of the more significant over the time scales of these experiments being phosphodiesterase inhibition (Oestreich, Malik et al. 2009) and activity at the A2A adenosine receptor, which may in itself have a cardioprotective role (Murphy and Steenbergen 2008, Pereira, Ruiz-Hurtado et al. 2012). Further, the concentrations used in these studies have been determined based upon previous

work, and used throughout for consistency and comparison. It would be interesting to perform dose-response studies to determine to what extent these responses described are maintained across a range of concentrations. Time constraints prevented the extension of scope to permit this.

8.2.5 Limitations with Mitochondrial Isolation Experiments

There were several drawbacks to the experimental techniques used in these experiments. From a technical perspective, there was no independent marker of the quality of Langendorff perfusion. In performing functional Langendorff experiments, I would usually measure both intraventricular pressure vs. time and creatine kinase activity before and during reperfusion. These are markers of injury, and of functional recovery, but can be used independently to identify outlying experiments where coronary perfusion is sub-optimal. This was not possible using the chosen experimental protocol, and so I cannot be certain that all the included data is from hearts that were very well perfused.

The absolute magnitude of the changes observed in the immature heart groups was low vs. the size of the initial absorbance- there is thus a low signal to noise ratio. This is reflected in the relatively large values for SEM seen in these results. Alternative measures of mitochondrial function may be less susceptible to this problem; for example the calcium retention assay performed by other groups (Andrienko, Pasdois et al. 2017) may be a more sensitive assay in this context. Also, there is no direct measure of the integrity of the mitochondria isolated; it is assumed that they are functional, as they exhibit a swelling response to Ca^{2+}, but the proportion of intact vs damaged or ruptured mitochondria is not clear; this conceivably could even account for the relatively low response of the P14 mitochondria. Direct visualisation by electron microscopy would be a reasonable way of quantifying the quality of the mitochondrial isolates.

8.3 Ongoing & Further Experimental Studies

Each strand of this work leaves unanswered questions and alternative approaches to the problems studied.

The proteomic work provided strands for further investigation described in this thesis. However, it produced a very large data set with a scope much larger than the networks immediately surrounding cAMP signalling; this dataset could not be adequately explored whilst investigating the central hypothesis of this thesis. However, there are ancillary signalling networks that play intersecting roles with survival after cardiac insults; work investigating these networks and how they interact with the cAMP- related effects would provide fuller insights into the mechanisms of cardioprotection. Also, the variation in the proteome is not limited to relative changes in protein- level expression. A range of post- translational modifications take place; most notably regulation by phosphorylation at differing sites on proteins; but also a range of more recently recognised modifications. This additional layer of modification may well be important in regulation and control of the functions of these networks of proteins.

A significant finding from the mitochondrial work also was the demonstration of the influence of isoprenaline on MPTP opening without any intentional injury to the heart prior to isolation. That the MPTP may have a physiological role which alters in response to adrenergic signalling is important. A demonstration of this phenomenon in *in vivo* experiments is necessary in order to persuasively demonstrate that this observation is not an artefact of isolation; as it has significant consequences for understanding of the dynamic role that the mitochondrion may play in the overall function of the cardiomyocyte.

These observations, and those concerning the amelioration of MPTP opening after I/R, were at a receptor level only made with isoprenaline. This is a non-selective β-adrenergic agonist; and so any conclusions made about the function of this drug in this context do not discriminate between any of the β- receptor subtypes. Selective

β- receptor agonists are in use experimentally, and in everyday clinical practice so are readily available, with dobutamine, for instance a well characterised β_1 receptor agonist already widely used for its inotropic properties.

As these findings are relatively novel, they should also be validated through alternative experimental approaches. Similar experimental designs but involving measurements of mitochondrial membrane potentials, Ca^{2+} retention, and validation using the [^{3}H]2-DG method would provide replication of these findings using an alternative approach, and also some mechanistic insight.

The aim of the whole heart studies was to demonstrate the cardioprotective efficacy of the combination of Epac and PKA agonists; this was reaffirmed in the adult and demonstrated for the first time in the developing heart. This was shown in the context of a global ischaemic injury and subsequent reperfusion; this is used as a model for the kind of I/R injury sustained in cardiac surgery, with aortic clamping. However, this is a smaller problem than the burden of mortality and morbidity caused by regional ischaemia such as that seen with atherosclerotic plaque rupture leading to myocardial infarction. Although this is an extremely rare problem in the developing heart, for the adult, a demonstration of a viable cardioprotective intervention would be extremely important.

Given that in the model of global injury, protection has again been demonstrated, it would be useful to attempt to replicate that in an animal model. However, the safety and toxicity of the synthetic agents used for the experiments in this thesis has not been established; this would have to be shown before examining the effects in models of disease. PKA and Epac have widely described, and varying biological effects in various tissues (Robichaux-III and Cheng 2018). Therefore the systemic use of these agents by, for example, intravenous use is likely to lead to significant non-intended physiological effects. More targeted administration, for instance, by direct intracoronary injection as part of cardioplegia solutions may avoid some of the off-target effects; there are well- established large animal models in Bristol which could be adapted for these studies.

9 References

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